FORMULATION DEVELOPMENT OF BIODEGRADABLE IMPLANTS FOR EXTENDED PARENTERAL DELIVERY OF PROTEIN DRUGS

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Disputation am 18.February 2011

To My Mother

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CONTENTS

CHAPTER

1 Introduction

1.1 Protein Delivery

Insulin was the first therapeutic protein introduced, in the 1922 (Banting et al. 1922). Soon after, extensive investigations were started on delivery of insulin from different routs of administrations (Brown 2005). In addition to subcutaneous injection, rectal, intestinal, intratracheal, peritoneal, vaginal, scrotal sac, oral, dermal, pulmonary and nasal routes have been examined with various degrees of limited success and mostly failure (Jensen 1938). Currently, protein drugs are the most rapidly growing segment of biopharmaceutical drug market (Reichert & Paquette 2003). This is partly a consequence of the recombinant DNA technology which has led to an increased number of approved biotechnology medicines (Frokjaer & Otzen 2005).

Protein drugs are usually administered via parenteral or subcutaneous injections to circumvent their inherent instability in the gastro-intestinal tract as well as their low permeability across biological membranes (Frokjaer & Otzen 2005). However, proteins have a very short half-life when administered systematically due to renal filtration and immune responses (Hermeling et al. 2004). Thus, a delivery device which can deliver protein drugs at a controlled rate into the target site, to bypass systematic circulation, will be beneficial.

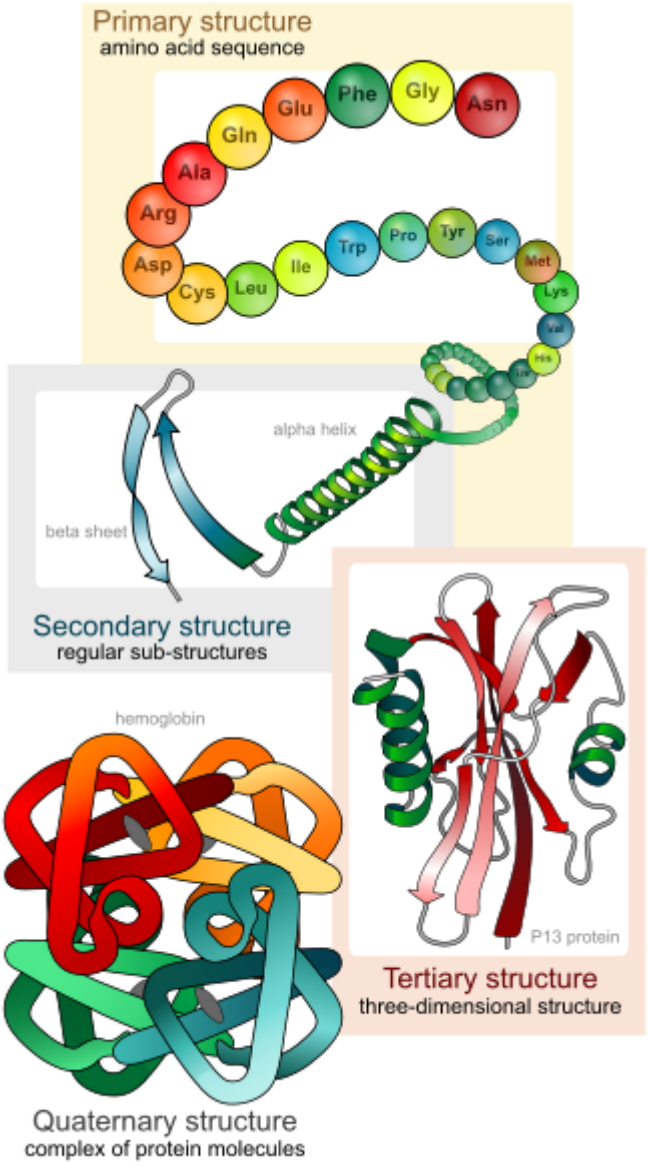

Figure 1.1 Levels of protein structure, from primary to quaternary structure (Picture taken from http://en.wikipedia.org/wiki/Protein_structure)

Proteins are polymers of amino acids (polypeptide). Amino acid chains with less than 40 residues are usually referred to as peptides. For proteins four distinct structural levels can be distinguished (Figure 1.1). The primary structure refers to the sequence of the different amino acids, held together by covalent bonds (peptide bonds). The secondary structure refers to highly regular local sub-structures. Two main types of secondary structure are the alpha helix and the beta strand. The tertiary structure presents a three-dimensional structure of a single protein molecule. The alpha-helices and beta-sheets are folded into a compact globule. This is driven by a number of non-covalent interactions and

hydrophobic packing (the affinity to the burial of hydrophobic residues from water). However, specific interactions such as salt bridges and disulfide bonds are necessary to stabilize the 3D structure. The quaternary structure is a larger assembly of several protein molecules. Many proteins do not have the quaternary structure and are active as monomers.

Proteins are the major players within the cells. To be able to perform their biological function, the specific conformation (3D structure) should remain intact. This will give proteins the ability to bind other molecules specifically and tightly. The best-known role of proteins in the cell is as enzymes which catalyze chemical reactions. In addition, many proteins are involved in the process of cell signaling and signal transduction, such as insulin. Antibodies are protein components of the adaptive immune system whose main function is to bind antigens or foreign substances in the body. Proteins can also take part in ligand transportation like haemoglobin. Receptors and hormones are highly specific binding proteins.

Moreover, the protein structure can trigger immune responses which can in consequence lead to a loss of therapeutic effect. However, the immunogenicity may consist of more serious clinical effects and toxicities. Aggregation is the most important structural change known to increase frequency of immune reactions (Moore & Leppert 1980; Hermeling et al. 2004). Unfolding and misfolding of proteins structure are other physical transformations which can present different epitopes than native proteins and result in immunogenicity (Gass et al. 2004). Thus, maintenance of protein molecular structure and integrity, referred to as protein stability, is the biggest challenge for safe and effective delivery of protein drugs.

Stability of protein drugs can be compromised, physically and/or chemically, during manufacturing of dosage form, storage and/or release in aqueous medium. External stress factors such as shear, pH, temperature, protein concentration, exposure to interfaces and dehydration can favor formation of unstable forms and/or aggregates. Small proteins may require harsh conditions to unfold whereas large and/or multiple unit proteins aggregation can be initiated under relatively gentle conditions (Frokjaer & Otzen 2005).

Proteins aggregation can be physical, through non-covalent association without changes in the primary structure, or be chemical by formation of new covalent bonds. Proteins can undergo both mechanisms simultaneously and form soluble and/or insoluble aggregates. Environmental factors such as temperature, pH and ionic strength as well as exposure to hydrophobic surfaces, organic solvents and metal ions have been shown to affect protein aggregation. Processing conditions such as shaking, shearing and pressure can also induce proteins aggregation. Additionally, the reconstitution step may also generate protein aggregates (W. Wang 2005).

Chemical degradation involves the primary structure of proteins. This includes deamidation, amide bond cleavage, acylation, oxidation and disulfide bond exchange. Deamidation of asparagine and glutamine can occur through both acid or base catalyzed mechanisms (Houchin & Topp 2008). In acidic conditions, deamidation proceeds via direct hydrolysis of the side-chain amide bond, through a nucleophilic attack of water on the amide carbon. Hydrolysis of amide bonds is similar to ester hydrolysis but is two orders of magnitude slower, in aqueous solution. In basic solutions, deamidation involves the intramolecular nucleophilic attack of the backbone amide nitrogen on the side-chain amide carbon. Deamidation in polymer matrices, however, favors the intramolecular pathway, regardless of the effective pH in the polymeric system (Song et al. 2001). This change in the mechanism is suggested to be due to limited water available as a reactant in such formulations.

Amide bond cleavage is often observed after aspartic acid (Asp) residues. This reaction is governed by acid catalyzed intramolecular Asp side chain. Thus, the reaction was not seen above the pK_a of aspartic acid side-chain (pK_a=3.9) (Houchin & Topp 2008). This is considered as a common source of instability for peptide.

Acylation can occur by nucleophilic attack of encapsulated protein to reactive sites of carrier polymer. This was first reported between small amines and polyesters. Acylation of peptides in microspheres based on poly(lactic-co-glycolic acid) has been reported both *in vitro* and *in vivo*. Nucleophilic functional groups including primary amines (Lucke et al. 2002) and hydroxyl groups (Na et al. 2003) were suggested as the potential reactive sites. Addition of water-soluble divalent cationic salts was shown to inhibit the acylation

of peptides in PLGA microspheres (Zhang et al. 2009). PEGylation of a polypeptide was also shown to decrease acylation because of the steric hindrance of the PEG strand (Na & DeLuca 2005).

Oxidation is another reported source for chemical degradation of proteins. Cysteine and methionine are most sensitive to oxidation. There are contradictory data on the pH-dependency of this reaction (Shacter 2000; Bach et al. 1994). Nevertheless, oxidation rate may increase if the microclimate pH induces protein unfolding resulting in greater exposure of reactive amino acids to the solvent (J. Chu et al. 2004).

Disulfide bond exchange, which involves a nucleophilic attack of a free thiol group on a disulfide bond, has also been shown for bovine serum albumin encapsulated in polymeric matrices (Crotts & T. G. Park 1997). This reaction will lead to covalent aggregation of the protein.

Conformational stability of proteins is inversely related to their hydration level, being higher in the solid state (Hageman 1988; Bell et al. 1995). A certain level of hydration is necessary to afford proteins the flexibility for function but this flexibility also develops into large-scale collective motions which can lead to unfolding. Similarly, chemical reactions proceed at a much lower rate in the solid than in the dissolved state (Chien-Hua & Yuan-Yuan 1998).

A solid protein, as formed by lyophilization or spray drying, is generally in the amorphous state. In this state, protein molecules exhibit many of the dynamical features of polymeric glass forming systems (Hill et al. 2005). Proteins fall into the category of ‘‘strong’’ glass-formers (Fan et al. 1994) which exhibit very small changes in heat capacity (C_p) at their glass transition temperature (T_g), often making it difficult to observe the T_g when using differential scanning calorimetry (DSC) measurements. With conventional DSC, at first heating scan protein exhibits no discontinuity reflective of a T_g up to the characteristic thermal denaturation endotherm at melting temperature (T_m). When the denatured protein samples are heated in the second scan a clear glass transition appears, usually close to T_m. For example, T_g and T_m of bovine serum albumin are 195°C and 220°C, respectively. The absent of T_m in the second scan confirms denaturation of the

heated protein sample above melting temperature. Presence of water lowers both T_m and T_g. Thus, it was concluded that a change in C_p (i.e. T_g) only can be measured when the protein is in the unfolded or aggregated state (Hill et al. 2005). However, recent development of high ramp rate DSC provided greater sensitivity in terms of heat flow which made it possible to measure glass transition temperature at first DSC scan (Katayama et al. 2008).

1.2 Biodegradable Materials

To achieve controlled release of drugs administered via parenteral route, various approaches have been attempted: high viscosity products, complex formation, drug suspension instead of drug solution and oil solution instead of aqueous solution and subcutaneous implantation of drug pellets. Although these methods may still be used for achieving controlled release parenteral products, the newer methods make use of biodegradable and non-biodegradable polymers for this purpose. The use of biodegradable polymers avoids the need for removal of the device.

Biodegradable polymers can be either natural or synthetic (Mishra et al. 2008). Natural ones include polysaccharides and protein-based polymers, such as albumin, collagen and gelatin (Mohanty et al. 2000). Synthetic polymers provide with a wider range of mechanical properties and degradation rate. Polyesters such as poly(lactic acid), poly(glycolic acid) and their copolymers (PLGA, Figure 1.2) (Holland et al. 1986) as well as poly(ε-caprolactone) are the most commonly used polymers in protein formulations (N. H. Shah et al. 1993). In addition, other polymers such as polyphosphoesters are being investigated for protein and gene delivery (Zhao et al. 2003; Caliceti et al. 2000; Andrianov & Payne 1998). Poloxamers, copolymers of polyethylene oxide and polyoxypropylene, are another interesting class which provided a wide range of applications in pharmaceutical and biomedical field and were investigated for delivery of proteins (Kwon & Okano 1999).

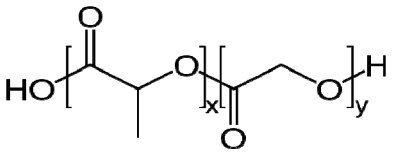

Figure 1.2 PLGA chemical structure (Picture taken from http://en.wikipedia.org/wiki/PLGA)

PLGA is synthesized by means of random ring-opening co-polymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid (Figure 1.3). Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained. Amorphous PLGAs show a glass transition temperature in the range of 40-60°C.

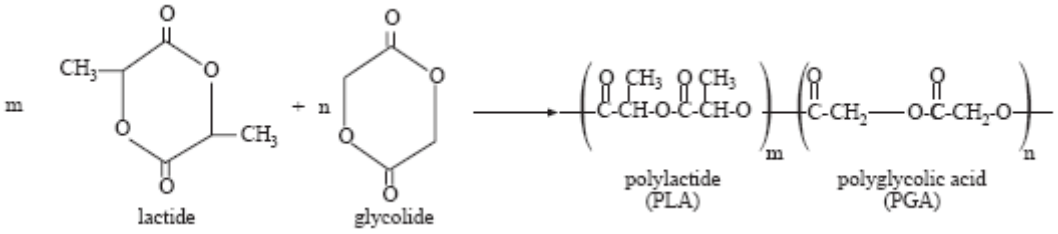

Figure 1.3 PLGA synthesis by random ring-opening co-polymerization of lactide and glycolide (Deniz 1999)

PLGA degrades by bulk erosion (1-6 months) through hydrolysis of the ester bonds (van De Weert et al. 2000) to its monomers, lactic acid and glycolic acid. These monomers, under normal physiological conditions, are by-products of various metabolic pathways in the body. Therefore, there is very minimal systemic toxicity associated with using PLGA for drug delivery or biomaterial applications. Depending on the need, degradation rate of PLGA can be tailored by the choice of PLGA type. The most important factors influencing the degradation rate are co-polymer composition and initial molecular weight (Alexis 2005). Copolymers with 50:50 monomers ratio exhibit the fastest degradation. In addition, polymers that are end-capped with esters (as opposed to the free carboxylic acid) demonstrate longer degradation half-lives. Additional components of formulations such as drugs and plasticizers can affect degradation rate directly through interactions with the polymer or indirectly via changing the matrix properties e.g. its hydrophilicity and porosity.

PLGA is the most commonly used biodegradable polymer for drug delivery (Shi & Li 2005). This can be ascribed in part to the approval of several PLGA-based drug-delivery devices by the FDA for human use. The first synthetic biodegradable (absorbable) sutures (Dexon), having been marketed in 1970 by Cyanamid, was prepared by melt extrusion of high molecular weight poly(glycolic acid). Next, poly(lactic acid) containing narcotic antagonists has been prepared as spherical beads by transfer molding and as cylindrical rods by extruding the melt (Schwope et al. 1975).

PLGA has been successfully applied for delivery of peptide-based as well as low-molecular weight APIs. Examples of commercial products based on PLGA are listed in table 1.1. They include mainly injectable microspheres for extended drug delivery over weeks to months (Gad 2008; P. G. Schmidt et al. 2007; Shi & Li 2005). However a safe and effective delivery of large globular proteins from PLGA-based systems is still a big challenge.

Table 1.1 Examples of marketed PLGA-based injectable therapeutics

Trade name	API	Company	Indication
Arestin®	Minocycline[1]	OraPharma	Periodontal disease
Decapeptyl®	Triptorelin[2]	Ferring	Prostate cancer
Decapeptyl® LP	Triptorelin[2]	Ipsen	Prostate cancer
Eligard®	Leuorolide[2]	Astellas	Prostate cancer
Enantone® Depot	Leuorolide[2]	Takeda	Prostate cancer, endometriosis
Enantone® Gyn	Leuorolide[2]	Takeda	Prostate cancer, endometriosis
Lupron® Depot	Leuprolide[2]	TAP	Prostate cancer
Leuprone® HEXAL®*	Leuprolide[2]	Hexal	Prostate cancer
Nutropin® Depot	Human growth hormone[3]	Genentech	Short stature
Pamorelin® LA	Triptorelin[2]	Ipsen	Prostate cancer
Parlodel® LA	Bromocriptine[1]	Sandoz	Pituitary tumors
Profact®*	Buserelin[2]	Sanofi-Aventis	Prostate cancer, endometriosis
Risperdal® Consta	Risperidone[1]	Janssen/Alkermes	Schizophrenia
Sandostatin® LAR	Octreotide[2]	Novartis	Acromegaly, carcinod syndrome
Somatuline® LA	Lanreotide[2]	Ipsen	Acromegaly
Suprecur® MP	Triptorelin[2]	Pfizer	Prostate cancer
Trelstar® Depot	Buserelin[2]	Aventis	Endometriosis
Zoladex®*	Goserelin[2]	Astra Zeneca	Prostate cancer

* Implant
1 Low MW drug
2 Peptide
3 Protein, discontinued

1.3 PLGA-Based Delivery Systems for Proteins

Biodegradable drug delivery systems based on PLGA can be in the form of microparticles or implants (Jain 2000) (Figure 1.4). Microparticles are often preferred to single unit implants because of easier administration. Solid implants are administrated by surgical intervention or by insertion into the subcutaneous tissue using large-bore needles (trocar). Microparticles, however, have preferentially a size of less than 250 µm (J. H. Park et al. 2005) which allow injection through smaller needles.

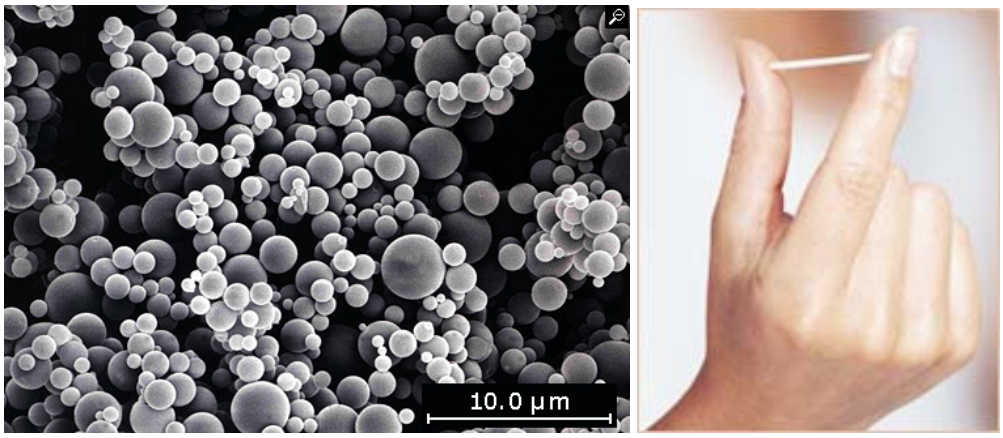

Figure 1.4 Microparticles (left) and solid implant (right) (Pictures taken from http://www.alrise.de and http://healthyhabitswellness.net, respectively)

Microparticles can be prepared by different microencapsulation techniques including solvent evaporation/extraction, organic phase separation and spray drying (Jain 2000). However, during microencapsulation large interfaces can be formed (for example between the organic polymer phase and the aqueous protein phase) which is a common destabilizing factor for protein drugs. Indeed, interfacial adsorption can promote denaturation and aggregation of proteins (Pérez & Griebenow 2001). As well, hydrophobic interaction of PLGA with proteins during emulsification or co-dissolution with PLGA may lead to protein unfolding and subsequent aggregation (van De Weert et al. 2000a). Moreover, it can compromise the secondary structure of proteins, leading to partially unfolded and aggregation-prone conformations (Prestrelski et al. 1993). Instability of proteins during microencapsulation has been addressed by several studies (Kokai et al. 2010; Taluja & Bae 2008; Ghaderi & Carlfors 1997; Pérez et al. 2002). In addition, encapsulation efficiency is another challenge in microencapsulation of protein

drugs which are often very costly. Thus, development of clinically successful long-term protein delivery systems based on PLGA requires further improvement of production processes to optimize the drug loading efficiency as well as protein stability.

The only commercialized PLGA-based protein delivery device was Nutropin Depot® (Alkermes and Genentech) which was approved in 1999 by the US FDA. A single injection of this microsphere product provided sustained release of human growth hormone over 2 or 4 weeks (Brown 2005). Human growth hormone (hGH) is a 191 amino acid protein with a molecular weight of 22,125 Da. An anhydrous process was developed (ProLease®, Alkermes) to avoid prolonged contact of the protein with organic solvent (Cleland et al. 1997) or the high temperature during conventional spray-drying. In this method solid protein is dispersed into a PLGA solution and then ultrasonically sprayed into frozen ethanol overlaid with liquid nitrogen. However, the high process costs caused the commercialization of Nutropin to be discontinued in 2004 (Shi & Li 2005).

Solid implants, in contrast to microspheres, can be prepared with solvent-free processes. These include melt compression, injection/compression molding and melt extrusion. Such methods can avoid potential exposure of the dissolved form of proteins to surfaces and interfaces. Additionally, proteins are more stable, both physically (Hageman 1988; Bell et al. 1995) and chemically (Chien-Hua & Yuan-Yuan 1998), in solid state. Hence, it can be expected that manufacturing of protein-PLGA DDS in the solid state provides better stability of proteins during manufacturing.

1.4 PLGA Implants Prepared with Hot-Melt Extrusion

Hot-melt extrusion (HME) is a single-step process which makes it simple, efficient and continues and thus, cost-effective. It can result in large drug loadings, avoids water and organic solvents and does not require additional excipients such as surfactants. The melt extrusion process is capable of handling active agents of different particle sizes as well as amorphous solids or other polymorphic forms leading to the same product (Breitenbach 2002). Extrusion produces matrices by forcing formulation materials through a die and results in products with uniform shape and density. Solid molecular dispersions of drugs

in a polymeric matrix are usually obtained which increase remarkably bioavailability of poorly soluble drugs (Leuner & Dressman 2000). From the marketed PLGA-based injectable products (Table 1), a few are implants all of which are processed with extrusion. For example, Zoladex® has implantable cylinders (1/1.5 mm Ø, 10 mm length) based on PLGA 50:50 with tow doses of 3.6 mg and 10.8 mg for 28 days and 3 months, respectively.

Extrusion Process

General steps in preparation of PLGA implants with extrusion technique are depicted in figure 1.5. Prior to processing PLGA particle size can be adjusted by cryo-grinding or dissolution in a solvent with subsequent drying. This can decrease variability within a batch as well as batch-to-batch variability (Shiah et al. 2006). PLGA can be plasticized prior to the process. This can reduces glass-transition temperature of the polymer and thus improve the processability. As well, the plasticized polymer can be extruded at lower temperature which means less thermal stress for formulation excipients. Examples of such plasticizers include polyethylene glycol, triethyl citrate, glycerol and ethanol. For an efficient plasticization, the PLGA-plasticizer mixture was usually extruded followed by grinding. For plasticization of PLGA with ethanol, the polymer was added to ethanol (0.12 g/ml) at 45°C. A viscous gel was obtained which was dried to desired ethanol content. This was then extruded and finally ground to achieve pre-plasticized PLGA powder (Mauriac & Marion 2006). Some criteria in selection of plasticizer type include its permanence in the formulation especially during process and storage, biocompatibility and its effect on stability and release of API.

Drug powder preparation steps can include freeze-drying, milling and sieving to obtain desired particle size. In addition to particle size, water content of the drug powder should be controlled before processing.

Drug and PLGA powder should be blended in a way to achieve a homogenous mixture but at the same time to ensure drug stability. A solvent-free option is to mix drug and PLGA in powder forms in which the particle size of raw materials and humidity have influence on efficiency of mixing. Another possible approach is “cryogenic co-grinding” (Schutz & Freudensprung 2004). Raw materials in form of powder or granules are cooled

together below the glass-transition temperature of polymer(s) and mixed in a cryogenic mill. Another technique for blending involves addition of polymer powder into an aqueous drug solution and subsequently vacuum-drying (Deghenghi 1998). In processing of some commercial products, like Zoladex®, the peptide and PLGA were co-dissolved in a solvent (acetic anhydride) and subsequently freeze-dried with a rapid cooling to achieve good PLGA-peptide homogeneity (Hutchinson 1994). In order to remove the solvent, other techniques such as film casting or vacuum-drying can also be applied.

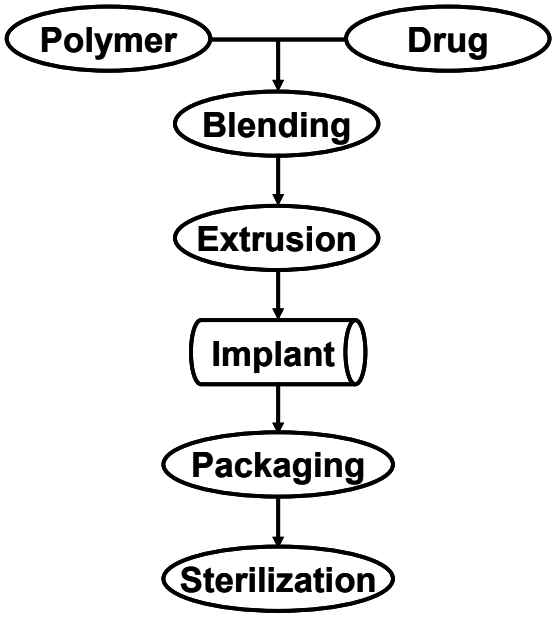

Figure 1.5 General steps in processing of PLGA implants with extrusion technique

Common extruders are either screw-extruders which use shear forces and pressure to convey material or ram-extruders. A ram extruder (Figure 1.6) applies a high pressure in the range of hundred bars. The pressure inserted, with a plunger, on the molten material in the barrel will affect density of implants. Residence time in the barrel as well as temperature can influence stability of formulation components. Finally, geometry of the die will define implants shape.

A screw-extruder (Figure 1.7) consists of a drive system, an extrusion barrel, rotating screw(s) and an extrusion die for defining product shape. The extruder can have either single- or twin-screw configuration which, in industrial scale, is commonly modular (Figure 1.8). It is possible to include feeding ports at different stages within the barrel which permit development of intricate dosage forms and applicability of HME for more sensitive APIs (Repka et al. 2008).

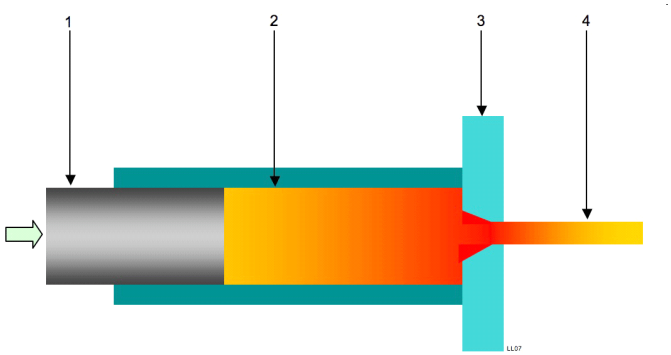

Figure 1.6 Schematic representation of a ram extruder; (1) a plunger, (2) barrel, (3) die and (4) extrudate

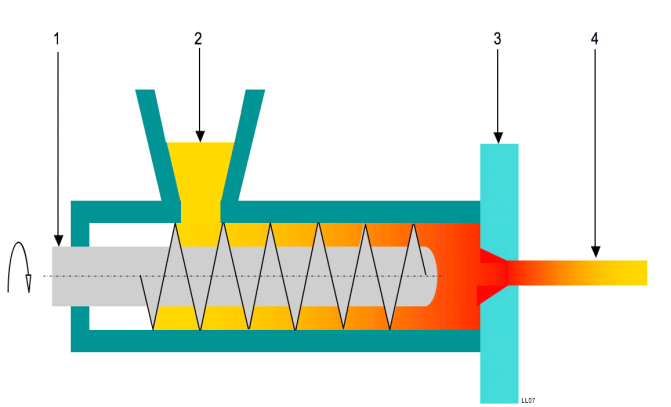

Figure 1.7 Schematic representation of screw-extruder; (1) screw, (2) feeding into barrel, (3) die and (4) extrudate

Twin-screw extrusion offers better mixing capability than single-screw extrusion. It can be used for in-situ melt-blending of raw materials. Moreover, it provides shorter residence times and the ability to combine separate batch operations into a single continuous process, thus increasing manufacturing efficiency (Andrews et al. 2009).

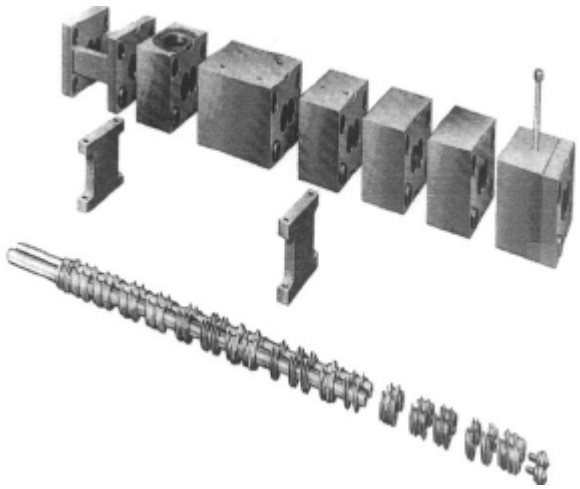

Figure 1.8 Typical array of modules and screws for a twin-screw extruder (Picture taken from http://americanpharmaceuticalreview.com/images/Article/Alvarez1.bmp)

Influencing parameters in screw-extruders are more complex than ram-extruders. These include screw speed and design, feeding rate, temperature and residence time (in case of using bypass mode) in the barrel. For example, increasing processing temperature (or application of plasticizers) can decrease viscosity of the melt (Ding et al. 2006). This in a ram-extruder and under constant pressure will increase density of the product. In a screw-extruder, in contrast, a reduced melt viscosity under constant screw speed can decrease pressure inside the barrel and thus reduce density of the product.

PLGA reservoir systems (Figure 1.9) can be designed to provide zero-order release and reduced burst release. This can be achieved by solution-casting of a core implant or injection of core formulation into a ready membrane as empty tube. Alternatively, DURIN™ Technology, utilizes a co-extrusion technique. In this system, tow extruders are connected to a coaxial die thus (Figure 1.10), a membrane coats the core as it exits the die (Gibson et al. 2002).

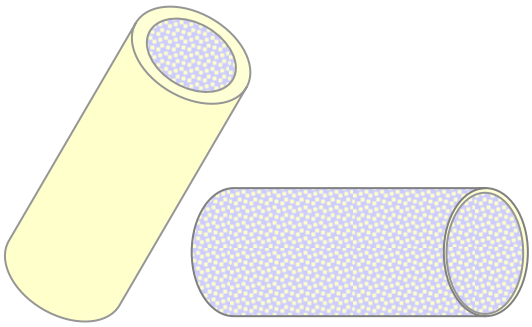

Figure 1.9 Reservoir- (left) vs. Matrix-type (right) implants

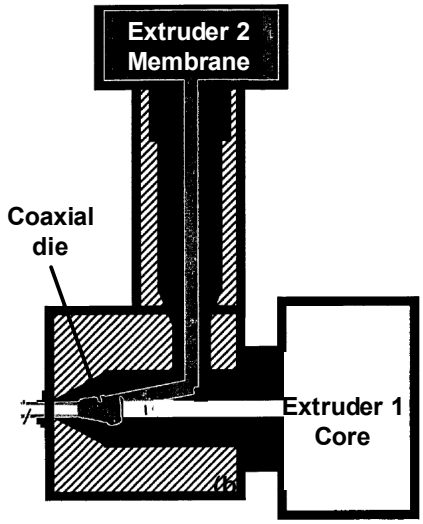

Figure 1.10 Schematic representation of co-extruder consist of tow extruders and a coaxial die (Gibson et al. 2002)

PLGA-leuprolide coaxial implants (Durect) are under clinical trials. The peptide exhibited zero-order release profiles in contrast to typical multi-phasic release of conventional PLGA implants. The release rate was decreased by increasing thickness of outer membrane.

Immediately after extrusion die, downstream auxiliary equipment can be utilized for cooling, cutting and/or collecting the finished product. Cylindrical implants are usually cut into ~ 10 mm rods and placed in syringes. The prefilled syringes (Figure 1.11) will be packed in heat-sealed, moisture (and light) proof, polymer/aluminum composite bags.

The dosage forms intended to be used in the body must be sterilized product. The steam sterilization can not be used because the high temperature can cause a deformation of the matrix and the penetrating high pressure steam can initiate a polymer hydrolysis (Vauthier & Bouchemal 2009). Gases sterilization is not recommended for implants because the residual vapor has been found to be mutagenic (Alexis 2005). Terminal sterilization with gamma-radiation is usually recommended. However, it can induce decomposition of the polymer. This effect is lower for lower molecular weight PLGA (Rothen-Weinhold et al. 1997) and under low doses of radiation (Sendl-lang et al. 2007). This random chain scission of PLGA and thus reduction of its molecular weight can affect the release especially in the erosion-controlled phase (Rothen-Weinhold et al. 2000).

Figure 1.11 Zoladex® pre-filled syringe (SafeSystem™)

Characterizations

HME produces PLGA implants with smooth surfaces and homogenous matrices without pores (Witt et al. 2000) (Figure 1.12). For example, densities of intraocular implants were 1.30 and 1.06 g/cm^3 when prepared with melt extrusion and solvent extrusion techniques, respectively (Zhou et al. 1998). The higher density of HME implants, e.g. compared to compressed tablets, can reduce drug release in the initial diffusion-controlled phase (Shiah et al. 2006).

PLGA based melanotan-I implants were produced by HME (Bhardwaj & Blanchard 1998). The in vitro release of the peptide exhibited a tri-phasic profile with an initial rapid release followed by a secondary phase of slow release. A tertiary phase of rapid release commenced after 3 weeks, due to erosion of the polymer. The polymer erosion and degradation are considered as the factors influencing the drug release and are controlled by the physical properties of the polymer. For example duration of the secondary phase of release has been found to be directly proportional to the molecular weight of the

copolymer. The total release time as well as the duration of the secondary phase are both dependent to the monomer ratio in PLGA co-polymers (Sanders et al. 1986).

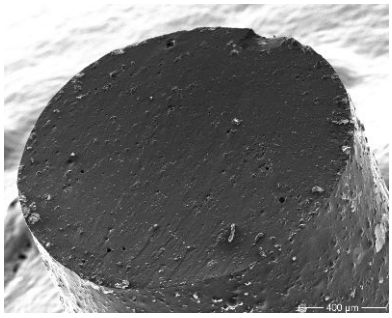

Figure 1.12 SEM image of PLGA implant prepared by HME

Degradation of PLGA implants usually shows a multi-phasic pattern. PLGA hydrolysis can start immediately upon contact with moisture, as in case of low molecular weight PLGA with free carboxylic acids groups. In case of end-capped PLGA, with comparably low acid numbers, however, the hydrolysis start after and induction period of at least 1 week (Witt et al. 2000). This induction period is the time needed for accumulation of enough free carboxylic acid end-groups to promote autocatalysis. Consequently, accelerated degradation phase starts. This second phase is attributed to the hydrolysis of the polymer chain, which occurs by random scission with a pseudo-first-order kinetics (Kenley et al. 1987).

Mass loss profiles of PLGA implants were characterized by a lag phase and a subsequent rapid erosion. Both the degradation rate and mass loss of PLGA implants were independent of the device geometry (surface/volume ratio). Interestingly, implants based on ABA triblock copolymers, consisting of poly(lactide-co-glycolide) A-blocks and poly(oxyethylene) B-blocks, exhibited a geometry-dependent mass loss (Witt et al. 2000). However, degradation rate of ABA copolymers was independent of geometry and slower than that of PLGA. Thus, the higher mass loss of the devices with higher surface/volume ratio was attributed to a facilitated out-flux of water-soluble degradation products immediately after polymer chain cleavage. SEM micrographs of swollen implants demonstrated the swelling of the ABA matrices (130% vs. 15% for PLGA implants), leading to cracking upon release of PEO.

In addition to polymer erosion, drug release from PLGA implants is also dependent on drug content, polymer and drug particle size (Zhou et al. 1998), porosity of the implants and size of the dosage forms (Viitanen et al. 2006).

Drug release from implants prepared with ram extrusion technique showed dependency on the polymer particle size (Hsu et al. 1994). Micronization of PLGA reduced release rate of the drug (isoniazid). This was attributed to a decrease in matrix porosity in that particle compaction facilitated the merging of polymer grains.

Increasing drug loading can increase the release of PLGA implants (Hsu et al. 1994). Presence of water-soluble drugs can also contribute in porosity of the matrix. In case of water soluble drugs, increasing drug loading increases porosity of implants upon release (Zhou et al. 1998). For perfectly immiscible drug-polymer systems, the percolation threshold is ~ 20-25% (Siegel 1989). The higher drug contents, on the other hand, increase probability of appearance of drug particles on or near the implants surface and thus can increase initial burst release (Zhou et al. 1998).

Biodegradable gentamicin sulphate loaded PLGA implants were produced by HME and showed equivalent release profile to Septopal® (Biomet Merck BioMaterials GmbH, Darmstadt, Germany) which consists of non-biodegradable polymethylmethacrylate (PMMA) beads loaded with gentamicin sulphate (Gosau & Müller 2010). Another example of PLGA-based implants for delivery of low molecular-weight drugs is ZT-1, a derivative of huperzine A for Alzheimer treatment which was processed with single-screw extruder at 70-90°C and currently is in phase II clinical trials.

Peptides own chemical and physical properties which make special difficulties in the processing and delivery of these drugs. A long-acting poly(lactic acid) implant containing vapreotide, an octapeptide, was prepared by hot-melt extrusion (Rothen-Weinhold et al. 2000). This led to formation of lactoyl lactyl-vapreotide conjugate during processing in presence of residual lactide in the polylactic acid. This illustrated the importance of carrier purity. Besides, in the utilized process the peptide/polymer mixture remained at 120°C for 1 h which might explain the peptide degradation even in absence of residual lactide.

However, protein exposure to high temperature and shear force or high pressure can potentially cause unfolding, even in the dry state, leading to irreversible aggregation or covalent modifications of proteins (Rothen-Weinhold et al. 2000). This might explain the very few repots on processing of protein formulations with HME (Ozkan et al. 2009). In this single report, hot-melt extrusion was used to process protein-encapsulated tissue engineering scaffolds based on polycaprolactone.

1.5 Characterization of PLGA-Protein Implants

The high complexity of protein therapeutics requires combination of physicochemical, immunochemical and biological analytics to control product quality, stability of encapsulated proteins and its integrity during release (Gad 2008). Examples of such techniques are listed in table 1.2. In most cases, encapsulated protein should be extracted from the carrier prior to evaluations. This can be done by hydrolysis of PLGA in alkaline mediums or dissolution in a solvent which is non-solvent for the protein. The isolated protein can be then analyzed with mass spectroscopy methods such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) for integrity of proteins primary structure. The method was used to monitor peptide acylation during degradation of PLGA microspheres (Na et al. 2003). High-performance liquid chromatography (HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can help to discriminate monomeric proteins from their aggregated forms. Fourier transform infrared (FTIR) spectroscopy provides characterization of secondary structure of proteins while embedded in the polymeric carrier (Fu et al. 1999). This method was also applied to determine distribution and conformation of lysozyme in PLGA microspheres (van de Weert et al. 2000c).

Table 1.2 Analytical methods for characterization and quality control of pharmaceutical peptides and proteins (Gad 2008)

Methods	Indicated Usage/Checking		
	Identity	Purity	Potency
Physicochemical			
Chromatography			
Reversed-phase high-performance liquid chromatography (HPLC, RP-1)	+	+	−
Ion exchange	−	+	−
Affinity	−	+	−
Size exclusion chromatography (SEC)	−	+	−
Spectroscopy			
Infrared spectroscopy	+	+	−
Raman spectroscopy	+	+	−
Fluorescence spectroscopy	−	+	−
Ultraviolet/visible (UV/VIS) spectroscopy	+	+	−
NMR spectroscopy	+	+	−
Mass spectrometry	+	+	−
Circular dichroism (CD)	+	+	−
Matrix-assisted light desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry	+	+	−
Electrophoresis			
Capillary electrophoresis	−	+	−
SDS–polyacrylamide gel electrophoresis (PAGE)	+	+	−
Isoelectric focusing	−	+	−
Immunochemical			
Radioimmunoassay (RIA)	−	−	+
ELISA	−	−	+
Western blot	+	−	−
Biological			
In vivo assays	−	−	+
In vitro (cell culture) assays	−	−	+

In Vivo Release

Drug release from biodegradable delivery systems is governed by a combination of two mechanisms: drug diffusion and polymer degradation or erosion (S. S. Shah et al. 1992). Drug loading, polymer molecular weight (Diaz et al. 1999), copolymer composition and end-group modifications are critical factors affecting the release properties in vivo (Rothen-Weinhold et al. 1997) as well as in vitro.

Available data on in vivo-in vitro correlation of drug release from PLGA implants are limited to peptides (and low molecular weight drugs). One example is the comparison of in vitro release of buserelin-PLGA implants, prepared by compression molding, with their pharmacokinetic profiles (Schliecker et al. 2004). Implants, from which the peptide release could be described by the Higuchi model over the entire release period (4 weeks), showed a level A in vitro–in vivo correlation (IVIVC). Level A IVIVC represents a point-to-point correlation between the in vivo absorption profile and the in vitro release profile. If a level A correlation cannot be established level B correlation can compare the mean in vitro dissolution time of the formulation with either the mean residence time in the body or the mean in vivo dissolution time of the formulation. For implants with a combination of diffusion- and erosion-controlled drug release a level B IVIVC could be obtained.

In Vitro Release

Delivery of large globular proteins in PLGA-based DDS has been limited because of the irreversible inactivation of these therapeutic agents during encapsulation process, upon hydration by release medium and during release. While there are extensive repots attempting optimization of protein stability and release from PLGA microspheres, examples on PLGA implants are relatively limited.

Encapsulated bovine serum albumin (BSA) in PLGA implants (prepared by solvent extrusion) formed insoluble non-covalent aggregates and was hydrolyzed after incubation in a physiological buffer at 37°C for 28 days (G. Zhu et al. 2000). The acidic pH and intermediate water content existing in the polymer were suggested as major sources of encapsulated BSA instability. Hence, basic additives were co-incorporated in order to stabilize the protein. Basic additives could reduce dehydration-induced structural changes of BSA during solvent evaporation (> 90% native proteins) (G. Zhu & Schwendeman

2000). Additives could also increase BSA release in the initial (diffusion-controlled) phase. Although the total release after 28 days was similar with and without a base (magnesium hydroxide), but the soluble fraction of remaining protein was higher in presence of the base. This was ascribed to the neutralizing effect of the base. The reduced degradation rate of PLGA was also correlated to this neutralizing effect (reduction of autocatalytic degradation). However, as shown by the authors, addition of magnesium hydroxide increased water uptake and porosity of implants significantly.

Human growth hormone (hGH) implantable tablets based on PLGA were also prepared by direct compression to maintain the hormone integrity and stability (Santoveña et al. 2010). Extent of protein release in the initial phase was correlated to the drug content. However, a more significant increase was obtained in the diffusional release of tablets with smaller thickness.

In attempts to optimize protein formulations based on PLGA, low molecular-weight polyethylene glycols (PEG) are one of the most popular additives. They have a plasticizing effect on PLGA and show no adverse effect on protein structure and activity (Malzert et al. 2003). PEG can also stabilize proteins in PLGA formulations (Castellanos et al. 2005). In general, PEG can either accelerate the release via pore formation or slow it down by reducing diffusion through increased viscosity. The increase of the release is usually during the initial burst and the diffusion-controlled release phases (Jiang & Schwendeman 2001; Kang & Singh 2001). The blends of PLGA with PEG also resulted in an accelerated and continues release i.e. the release lag phase was eliminated (Cho et al. 2001). This was attributed to faster diffusion of BSA through the swollen phase of the hydrogel-like structure in the blend. However, comparison of data in literature can show that effect of PEG on protein release is dependent on the molecular weight of PEG (Taluja & Bae 2008; Bezemer et al. 2000).

In another study, BSA release from implantable wafers based on methoxy polyethylene glycol (MPEG)-PLGA diblock copolymers was evaluated (M. S. Kim et al. 2005). The implants were prepared by direct compression. BSA release was bi-phasic with an initial burst at the first day (up to ~ 55%) followed by a very slow/ no release phase. The total protein release, however, did not exceed 65%. The wafers with higher MPEG amount induced faster BSA release due to higher water absorption and formation of cracks.

Addition of small intestinal submucosa (SIS) to the formulations could enhance BSA release completeness without initial burst effect.

Accordingly, it can be concluded that the main challenges of PLGA-based protein delivery, i.e. protein stability, high initial burst release and release incompleteness, are still to be addressed.

1.6 Objectives

The purpose of this work was:

to evaluate feasibility of hot-melt extrusion for processing of biodegradable implants based on protein-poly(lactide-co-glycolide), primarily with regard to protein stability during manufacturing process,

to characterize protein release from PLGA implants with special emphasis on initial burst release and release completeness,

to investigate the reason for incomplete release of proteins from PLGA implants and

to provide ways to optimize the release pattern and increase release completeness accordingly.

CHAPTER

2 Materials and Methods

2.1 Materials

Model Proteins

Albumin fraction V (BSA), lyophilized hen egg white lysozyme (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); albumin from chicken egg white (ovalbumin), cytochrome C from equine heart (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

Table 2.1 Some characteristics of the utilized model proteins

	BSA[1]	Lysozyme	Ovalbumin[2]	Cytochrome C[3]
MW (kg/mol)	66	14	44.3*	13
Number of amino-acid	585	129	385	104
Number of disulfide bridges	17	4	1	2
Number of free Cysteine	1	0	4	1
Melting point (°C)	220	204	230	194.6
Isoelectric point	4.9	11	5.1	10.2
pH (10% in water)	7.1	3.3	6.2	8.2

1 Multi domain protein
2 Phosphorylated-glycoprotein
3 Haem protein
* Total molecular weight

Polymer

Poly(D,L-lactide-co-glycolide) (Resomer® RG 502, end-capped 50:50 PLGA, inherent viscosity 0.2 dlg^{-1}, Boehringer-Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany).

Plasticizers

Polyethylene glycol 400, 1500 and 4000 (Lutrol® E, BASF AG, Ludwigshafen, Germany); benzyl benzoate (Merck KGaA, Darmstadt, Germany).

Other Excipients

Acetonitrile (HPLC gradient grade), dithiothreitol, ethanol, guanidine hydrochloride, hydroxylamine hydrochloride, potassium dihydrogen phosphate, sodium hydroxide, tetrahydrofuran (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); acetic acid, ethyl acetate, sodium acetate, sodium azide, sodium dihydrogen phosphate, trifluoroacetic acid (Merck KGaA, Darmstadt, Germany); Micrococcus lysodeikticus (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), medium chain triglyceride (MCT, Fagron Ltd., Barsbüttel, Germany).

2.2 Preparation of PLGA Implants

Proteins were either manually ground with a mortar and pestle or ball-milled (40 min, 100 rpm, Retsch MM2000, Retsch GmbH & Co. K.G., Haan, Germany) to reduce its particle size (example in figure 2.1). Particle size measurements were performed using powder laser diffractometer (HELOS/Bf, Sympatec GmbH, Clausthal-Zellerfeld, Germany).

Incorporation of ethanol into PLGA powder was done according to Mauriac & Marison (Mauriac & Marion 2006). Briefly, ground PLGA powder was added to ethanol (0.12 g/ml) and stirred for 1 min at 45°C. The resulted viscous gel was dried at room temperature until PLGA with additional 20% weight was obtained. Next, the polymer was mixed with untreated PLGA (40:60) at -10°C followed by extrusion at 75°C. The extruded products were then ground manually at -10°C and further dried to achieve PLGA with ethanol content of 8% (additional weight to the original).

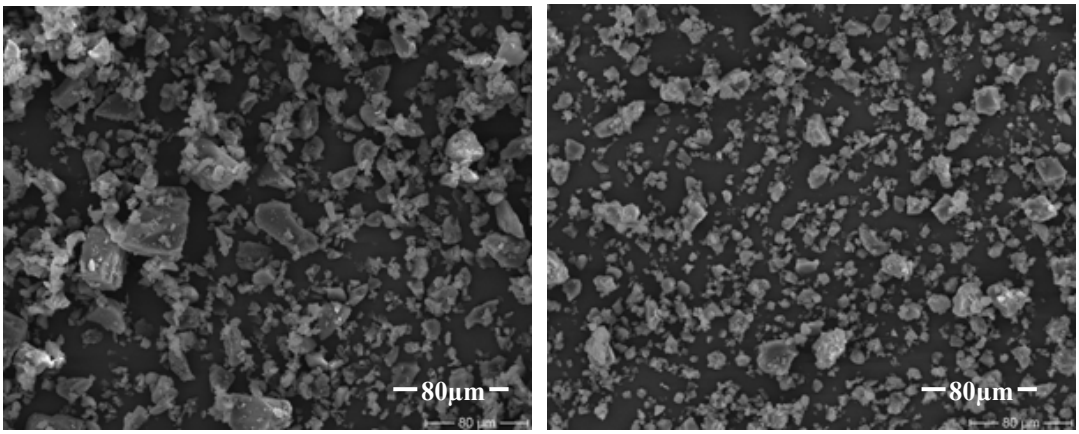

Figure 2.1 SEM pictures of BSA powder before (left) and after (right) ball-milling

For studies on pre-degraded PLGA, polymer powder was incubated in deionized-water in a horizontal shaker (80 rpm, 37°C; Gemeinschaft für Labortechnik, Burgwedel, Germany) for 14 days. The water was then discarded and the vacuum dried (24 h) polymer was ball-milled and used for melt-extrusion as described above.

Hot-Melt Extrusion with a Twin-Screw Extruder (HME)

The extrusion process was performed using a HAAKE MiniLab Rheomex CTW5 co-rotating twin screw extruder at 20 rpm screw speed. Powder blends (>4 g) of PLGA and protein were manually fed into the preheated barrel (90 – 105°C). A 1 mm cylindrical die was used resulting in matrices of 1.1 – 1.2 mm in diameter. Implant fractions were collected from the beginning, middle and end of the process. All experiments were performed with at least 3 replicates using one piece implant (~ 3 mm length and 3 mg weight) from each process fraction.

Hot-Melt Extrusion with a Syringe-Die Device (S-HME)

In order to have a higher throughput and less material use in HME formulation optimization, melt-extrusion with a syringe-die device was used as a screening tool.

Properly mixed formulation blends (~200 mg) of PLGA and protein (plus additive if mentioned) were charged into 1 ml polypropylene syringes (LUER LOK™, B-D®, Singapore). The syringes were fixed with a self-built die (Figure 2.2), having similar dimensions as the HME-die, and heated at 105°C in an oven for 10 min. The molten blends were then extruded manually, producing cylindrical matrices with diameters of 1.1

– 1.2 mm. The matrices were cut into 3 mm length (~ 5 mg) for recovery and release studies and 10 mm (~ 20 mg) for degradation study.

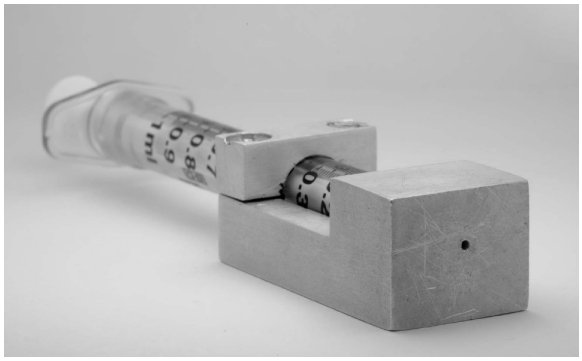

Figure 2.2 Syringe – die assembly used for S-HME

2.3 Protein Extraction from Implants

The implants (~ 3 mm) were dissolved in 1.5 ml ethyl acetate (protein nonsolvent / polymer solvent) and then centrifuged for 20 min at 25°C and 28,110g (Heraeus Biofuge stratos Haemo, Heraeus Instruments, Osterode, Germany) as described previously [28]. About 1 ml of the supernatant was removed and the washing cycle was repeated two more times. The protein precipitates were then dried under vacuum for 30 min (Heraeus oven VT 5042 EKP, Hanau, Germany, coupled with a chemistry hybrid pump, Vacuubrand GmbH, Wertheim, Germany) to remove residual ethyl acetate. The dried protein pellets were dissolved in 1 ml release medium. The concentration of soluble protein was quantified by BCA assay kit (Pierce, Rockford, USA). These protein solutions were also used to determine the concentration of active protein after extraction and also for HPLC. To study lysozyme recovery upon rehydration, implants were incubated in the release medium for one day before extraction.

2.4 Biological Activity of Lysozyme

The biological activity of lysozyme was measured with a modified turbidimetric assay [30]. The corrected linear rate of the absorbance decrease at 450 nm of a *Micrococcus*

lysodeikticus cell suspension in 66mM phosphate buffer (pH 6.24) at 25°C was used to estimate the concentration of active enzyme.

The initial absorbance of the filtered cell suspension was adjusted to values between 0.6 and 0.7. The aqueous lysozyme solution (100 µl) was added to 2.5 ml suspension of the bacteria. Turbidity was measured for 2 min using a diode array UV-spectrophotometer with a Peltier thermostatted cell holder (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA) equipped with a UV-Chemstation biochemical analysis software. The slope of the linear portion was used for the quantification of active lysozyme concentration in the sample based on a freshly prepared standard curve (0 – 30 µg/ml).

2.5 HPLC

Extracted lysozyme from implants was characterized for possible oxidation of the protein by HPLC (SCL-10A VP, Shimadazu, Japan) using a C4 reversed phase column (Eurosphere-100, 7 µm, 125 mm × 4 mm, Knauer, Berlin, Germany). The solvent system consisted of water/acetonitrile/trifluoroacetic acid (A: 100/0/0.1, B: 0/100/0.1, V/V). A linear gradient method was applied (0-11-12 min 18.5-59-18.5%B) at a flow rate of 2 ml/min for 14 min and a column temperature of 25°C. Samples (25 µl) were injected and chromatograms obtained with a diode-array UV-detector (SPD M-10A, Shimadazu, Japan) were quantified at 281 nm.

2.6 MALDI-MS

Extracted BSA from implants was characterized for possible degradation. Protein masses were analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. Sinapinic acid was used as the matrix and samples were spotted using the dried droplet technique. The mass spectrometer was operated in the positive linear mode. MS spectra

were acquired over an m/z range of 4,000–80,000 and data was analysed using FlexAnalysis® software.

2.7 Differential Scanning Calorimetry (DSC)

DSC-studies of protein powder or implants were performed with a DSC821^e (Mettler Toledo AG, Giessen, Germany) coupled with a Mettler TC15 TA-controller. Samples of ~10 mg were accurately weighed in closed 40µl aluminum crucibles. When measuring above 100°C, a pinhole was introduced into the lid for the escape of water vapor. DSC-scans were recorded using a heating rate of 20 K /min under nitrogen atmosphere. Thermographs were normalized for samples weight. Effect of DSC scan rate on the melting point (T_m) was investigated for lysozyme.

2.8 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR-spectra were generated with an Excalibur 3100 FTIR spectrophotometer (Varian Inc., Palo Alto, USA). The spectra from protein powder or ground implants (with mortar and pestle) were collected using a horizontal ATR accessory with a single reflection diamond crystal (Pike Miracle, Pike Technologies, Madison, USA). Sixty four scans at 4 cm^{-1} resolution were averaged and spectral contributions coming from water vapor in the light pass were subtracted using Varian software (Resolution Pro 4.0). Second derivative data were processed with the same software. Finally, all spectra were treated with a 13-point smoothing function.

Implants from released samples were vacuum dried before analysis. Ratios of PLGA:protein in released samples were estimated based on an FTIR peaks standard. Peak-height ratios of PLGA to amide I bonds were collected for physical mixtures of PLGA-protein with different ratio.

2.9 EDX-SEM

Protein distribution was examined by elemental mapping of the cross sections of implants for the characteristic X-ray peak of sulfur. The elemental distributions were investigated by scanning electron microscopy (Hitachi S-2700, Tokyo, Japan) combined with energy-dispersive X-ray (EDX) spectroscopy using a Röntec XFlash-SDD-detector. The implants were coated with carbon to make them conductive.

2.10 Density Measurement

In order to compare the porosity of implants, their apparent densities were estimated. Glass pipettes (1 ml) filled with medium chain triglyceride (MCT) (a nonsolvent for the implant ingredients) were used for volume measurement. Several pieces of implants from different fractions of the extrusion process were weighed and put together into the oil-filled pipette to achieve significant changes in the oil volume. Densities were calculated by dividing the total weight by the volume change.

2.11 Protein Release

Implants (3 mm long) were placed in screw cap sealed test tubes filled with 4 ml of release medium (one implant per vial, n=3). For lysozyme 33 mM pH 5 sodium acetate buffer containing 0.01% sodium azide, as preservative, was chosen according to the stability optimum of lysozyme (Claudy et al. 1992). For other proteins, 10 mM PBS pH 7.4 containing 0.01% (w/v) sodium azide, as preservative, was used as release medium. The vials were incubated in vertical position in a horizontal shaker (80 rpm, 37°C; Gemeinschaft für Labortechnik, Burgwedel, Germany). The release medium was replaced with fresh medium at each sampling time point. Protein concentrations in release samples were quantified by Micro BCA assay (Pierce, Rockford, USA) using a freshly prepared standard curve (0 – 20 µg/ml).

2.12 PLGA Degradation Study

Implants (10 mm long) were placed in screw cap sealed test tubes filled with 1 ml of 10 mM PBS pH 7.4 containing 0.01% sodium azide as preservative (one implant per vial, n=3). The vials were incubated in vertical position in a horizontal shaker (80 rpm, 37°C; Gemeinschaft für Labortechnik, Burgwedel, Germany). The release medium was completely removed with a pipette and replaced with fresh medium after first day and weekly afterwards. At predetermined time points degrading implants were withdrawn, vacuum dried for 24 h, dissolved in THF and analyzed for quantifying the mass and the molecular weight distribution of the remaining polymer.

The pH in the release medium was monitored with a pH-meter (Sartorius, Sartorius AG, Göttingen, Germany) at each sampling time point. The difference of the measured pH and the original pH of the release medium (pH 7.4) was used for the calculation of the cumulative pH changes (in %) during incubation.

2.13 PLGA Molecular Weight Determination

Gel permeation chromatography (GPC) analysis was carried out using a Shimadzu (Shimadzu, Tokyo, Japan) LD-10 liquid chromatograph equipped with degasser, pump, auto-injector and column oven in combination with a Viscotek triple detector (TDA-300, Viscotek, Malvern Instruments Ltd., Malvern, UK) operated in double mode (differential refractive index, viscosimetry). A column with a linear range from 500 g/mol to 18,000 g/mol (Mesopore 7.5 µm×300 mm; Varian Inc., Darmstadt, Germany) was used as stationary and THF as mobile phases. The sample concentration was 4% with the corresponding injection volumes of 25 µl. Column and detector were operated at 30°C and the flow rate was 1 ml/min. A universal calibration method (third-order polynomial fit, R^2: 0.99996) was applied to determine the molecular weights of PLGA, which was obtained from polystyrene standards with peak molecular weights of 580 g/mol, 1,260 g/mol, 2,360 g/mol, 4,920 g/mol, 9,920 g/mol, 19,880 g/mol (Varian Inc., Darmstadt, Germany). Data acquisition was performed using Omnisec software (Viscotek, Malvern Instruments Ltd., Malvern, UK).

2.14 PLGA Mass Loss

The mass loss of the implants in release medium was determined by GPC through quantification of the polymer mass at each sampling point. The refractive index detector served as concentration detector, with a calibration constant of the differential refractive index detector kcal of 30.588 mV, a refractive index of the mobile phase n_0 of 1.405 and a refractive index increment dn/dc of 0.054 ml/mg. The refractive index increment was thereby constant in the linear range of the column (500–18,000 g/mol). It should be noted that the polymer mass could be quantified only when PLGA was soluble in THF. The total polymer mass loss was thus reduced by irrecoverable polymer fractions and the study stopped when residual implant was not soluble in THF.

2.15 Uptake of Release Medium and PEG Release

Implants were weighed in the initial dry form (t_0). Medium uptake of implants was determined by their weight gain during release (Eq. (1)). At predetermined time points (t_i), the implants were removed from the release medium, blotted with tissue paper to remove surface medium and then weighed. The weight gain values were corrected for the amount of released protein. The studies were stopped when the implants turned into very soft matrices and could not be handled in a piece.

After 1 day release (t_1) the water content of S-HME implants (lysozyme:PEG:PLGA, 10:9:81) was measured using thermogravimetric analysis (TGA) with a Mettler TC15 TA-controller (Mettler Toledo). Total mass loss and the amount of released PEG were calculated according to Eqs. (2) and (3), respectively.

Weight gain (t_i) = weight wet (t_i) – initial weight (t_0) (1)

Mass loss (t_i) = water content (t_i) – weight gain (t_i) (2)

PEG release (t_1) = mass loss (t_1) – lysozyme release (t_1) (3)

CHAPTER

3 Results and Discussion

A

Improved Lysozyme Stability and Release Properties of Poly(lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion

The purpose of this study was to assess the feasibility of hot-melt extrusion for preparing implants based on protein/poly(lactide-co-glycolide) formulations with special emphasis on protein stability, burst release and release completeness.

3.A.1 Background

Maintenance of protein stability during formulation process, storage and release is one of the main challenges for the effective delivery of protein drugs. Growing attention has been paid to the parenteral delivery of proteins in biodegradable injectable delivery systems in order to protect them from degradation and to allow for their controlled delivery (Giteau et al. 2008a).

Poly(lactide-co-glycolide) (PLGA) has been successfully used as biodegradable carrier material in controlled release systems of low-molecular-weight drugs and peptides. The application of PLGA for the delivery of protein drugs, which have a much higher structural and functional complexity compared to small molecules and peptides, has been less successful. This is mostly due to incomplete recovery and release of native proteins.
In this study, hen egg white lysozyme, a glycosidase with a molecular weight of approximately 14.7 kDa and an isoelectric point around 11, was chosen as the model protein. Lysozyme is a popular model protein in pharmaceutical research and its incorporation into PLGA-based delivery systems has been reported extensively (Aubert-Pouëssel et al. 2004; Ghassemi et al. 2009; Giteau et al. 2008b; Kokai et al. 2010; E. S. Lee et al. 2007; Taluja & Bae 2008). Although lysozyme has sometimes been referred to be a relatively stable protein, its low recovery in the presence of PLGA was remarkable (Ghassemi et al. 2009; van De Weert et al. 2000a). The low recovery of lysozyme as well as its incomplete release from PLGA-based delivery systems has been related to the protein instability during manufacturing of the delivery system and during release (Aubert-Pouëssel et al. 2004; Giteau et al. 2008b; Diwan & T. G. Park 2001).

Biodegradable drug delivery systems based on PLGA can be in the form of microparticles or implants (Jain 2000). Microparticles, which are often preferred to single unit implants because of easier administration, can be prepared by different microencapsulation techniques including solvent evaporation/extraction, organic phase separation and spray drying (Jain 2000). However, for protein drugs, the formation of large interfaces during microencapsulation (for example between the organic polymer phase and the aqueous protein phase) is a common destabilizing factor because of interfacial adsorption followed by denaturation and aggregation of proteins (Pérez & Griebenow 2001). Additionally,

hydrophobic interaction of PLGA with proteins during emulsification or co-dissolution with PLGA may lead to protein unfolding and subsequent aggregation (van De Weert et al. 2000b). Additionally, it can compromise the secondary structure of proteins, leading to partially unfolded and aggregate-prone conformations (Prestrelski et al. 1993). Instability of lysozyme during microencapsulation has been addressed by several studies (Kokai et al. 2010; Taluja & Bae 2008; Ghaderi & Carlfors 1997; Pérez et al. 2002).

PLGA implants can be prepared with solvent-free processes such as melt compression, injection/compression molding and melt extrusion. Accordingly, these promising methods for protein formulations can avoid potential stress during the incorporation of a protein drug with regard to the exposure of its dissolved form to surfaces and interfaces. Conformational stability of proteins is inversely related to their hydration level, being higher in the solid state (Hageman 1988; Bell et al. 1995). Similarly, chemical reactions proceed at a much lower rate in the solid than in the dissolved state (Chien-Hua & Yuan-Yuan 1998).

Hot-melt extrusion is a single-step process which potentially offers many advantages for pharmaceutical applications over various microencapsulation processing techniques. It can result in large drug loadings, avoids water and organic solvents and does not require additional excipients such as surfactants. However, protein exposure to high temperature and shear force or high pressure can potentially cause unfolding even in the dry state, leading to irreversible aggregation or covalent modifications of proteins (Rothen-Weinhold et al. 2000). Accordingly, lysozyme was incorporated into PLGA implants by hot-melt extrusion to examine whether this solvent-free process can provide a better lysozyme stability during processing and also during release.

One of the biggest issues for protein delivery appears during rehydration of the protein upon contact of delivery systems with aqueous in vitro or vivo medium (Prestrelski et al. 1993). The increased protein mobility upon hydration and the close vicinity of the molecules can initiate (non-)covalent aggregation. Special emphasis was therefore put on characterizing the quality of lysozyme during the initial drug release phase in order to differentiate rehydration-induced changes from other potential changes, which can for example arise upon polymer degradation through acidification of the implant interior.

These instabilities can result in incomplete release of lysozyme (Ghassemi et al. 2009; Giteau et al. 2008b; Tae Gwan Park et al. 1998; van de Weert et al. 2000c).

Hydrophilic additives have been used to improve protein release from polymeric matrices. They can facilitate release of PLGA degradation products by increasing pore formation and hence prevent acidification of the matrix core. Low-molecular-weight polyethylene glycols (PEG) are hydrophilic additives with a plasticizing effect on PLGA. PEG generally increases the release during the initial burst and the diffusion-controlled release phases (Kang & Singh 2001; Jiang & Schwendeman 2001) and has no adverse effect on lysozyme structure and activity (Malzert et al. 2003). Therefore, PEGs with average molecular weights of 400 and 1500 Da were incorporated into lysozyme-loaded implants and their effects on lysozyme release were investigated.

3.A.2 Lysozyme Stability during Hot-Melt Extrusion Process

Protein instability during manufacturing of different PLGA delivery systems has been addressed by several studies (Ghaderi & Carlfors 1997; Pérez et al. 2002; J. Wang et al. 2004). In hot-melt extrusion, there are some stress factors (e.g., elevated temperature in combination with shear forces), which can potentially affect protein integrity via physical and/or chemical modifications and consequently can lead to their inactivation. Systematic studies about the stress factors exerted on protein drugs during hot-melt extrusion are still missing. Therefore, the physical and chemical stability of lysozyme after hot-melt extrusion with extruder (HME) at 105°C were evaluated.

Differential scanning calorimetry showed a denaturation temperature (T_m) of 204°C for both native lysozyme and lysozyme-loaded hot melt extruded PLGA implants (Figure 3.A.1). This is in agreement with previous results on native lysozyme in solid state (Elkordy et al. 2002). The presence of the melting peak in the DSC scan can indicate conservation of protein conformation after extrusion at 105°C (Hill et al. 2005). It should be mentioned that increasing scan rate from 10–20 K/min T_m of lysozyme changed from 202°C–204°C (see supplementary data).

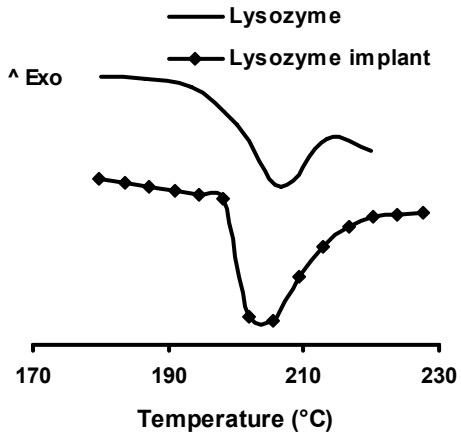

Figure 3.A.1 DSC-thermographs of lysozyme powder and 25% lysozyme-loaded PLGA implant produced by hot-melt extrusion at 105°C

Conformational stability of lysozyme in HME implants was confirmed by ATR-FTIR. The spectra of implants containing 25% lysozyme were comparable to the lyophilized lysozyme powder used to prepare the implants (Figure 3.A.2a). Thus, there was no indication for denaturation (shifts or distortion of bands) or aggregation (intermolecular β-sheet formation) as a consequence of the exposure to elevated temperature and pressure during the extrusion at 105°C based on these data. The second derivative spectra of the Amide I band (Figure 3.A.2b) suggested a negligible red-shift of the bands above 1660 cm^{-1}, assigned to turns and β-sheet (van de Wert et al. 2000c), which can be caused by subtraction of PLGA background or small loosening of the turn structures.

Lysozyme was completely recovered (99 ± 1.1% based on initial loading) from the implants showing 98 ± 5.9% biological activity. HPLC analysis of extracted protein showed that no oxidation of lysozyme occurred during the HME process. Chromatograms showed the same peak ratio of native (retention time 7.0 min) to oxidized lysozyme (retention time 6.7 min) as freshly prepared solution of unprocessed lysozyme powder (data not shown). In conclusion, lysozyme can be incorporated into PLGA-implants in its active form.

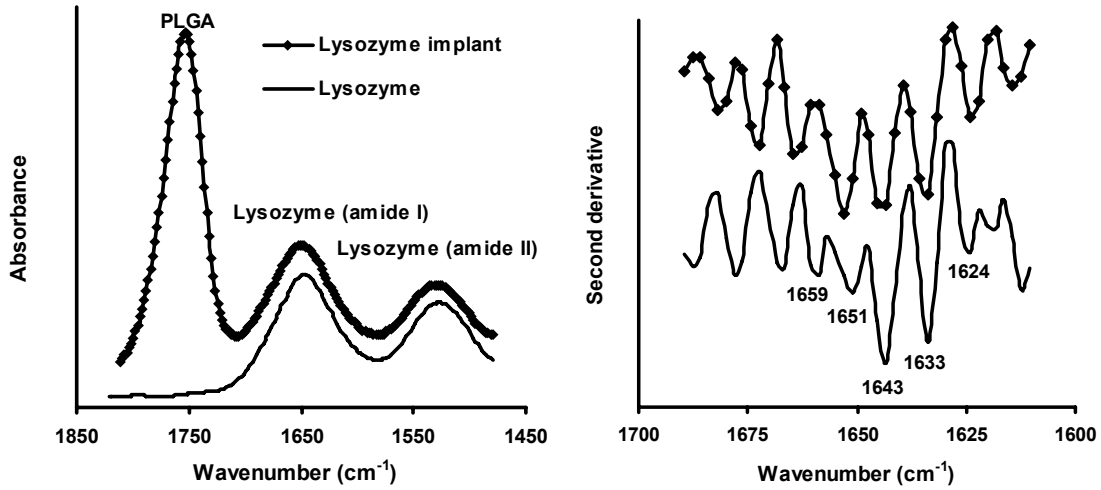

Figure 3.A.2 Lysozyme secondary structure by ATR-FTIR. (a) Spectra of lysozyme powder and ground PLGA implant containing 25% lysozyme produced by hot-melt extrusion at 105°C; (b) Second derivative spectra of Amide I region

3.A.3 Lysozyme Release

Lysozyme Recovery upon Contact with Release Medium

Moisture-induced degradation/aggregation is one major reason for the incomplete release of proteins (Schwendeman et al. 1997). Therefore, the effect of the initial hydration on protein recovery from the implant was examined after one day release.

Lysozyme was fully recoverable from the HME implants produced at 100 and 105°C after one day release. For implants prepared at 105°C, approx. 42% of the totally recovered amount was released within one day (burst release) (Table 3.A.1). The unreleased fraction (approx. 58%) of total protein remained in the fully active form in the implant. However, the released fraction showed some activity loss which summed up to an overall activity of 84%. Considering the native character of lysozyme found in the dry implants, the perturbation appears to occur during the rehydration step upon contact with aqueous release medium.

Table 3.A.1 Effect of process temperature on lysozyme recovery from PLGA implants containing 25% lysozyme produced with hot-melt extrusion

	HME at 105°C	HME at 100°C
Released – day 1 (%)	41.9 ± 2.9	26.5 ± 2.4
Extracted (%)	58.1 ± 0.2	73.6 ± 1.4
Total recovery (%)	100.0 ± 2.9	100.1 ± 2.3
Activity of released fraction (%)	62.5 ± 1.7	92.5 ± 2.1
Activity of extracted fraction (%)	98.8 ± 1.1	99.2 ± 0.7
Total activity (%)	83.6 ± 1.8	97.5 ± 2.4

Interestingly, no enzyme activity was lost when the hot-melt extrusion was conducted at 100°C. This observation coincided with a lower burst release from 100°C HME-implants (27% vs. 42%) compared to the 105°C ones. Reduction of process temperature from 105°C to 100°C increased the density of the matrices from1.04 gcm^{-3} to 1.45 gcm^{-3}, respectively. A lower process temperature can increase the density of the product because of a higher melt viscosity (Ding et al. 2006). In fact, the higher melt viscosity results in a higher pressure (Akdogan 1996) and thus a lower free volume (Warfield 1966).

Elemental mapping of implant cross-sections showed an overall homogeneous distribution of lysozyme particles in implants produced at 105°C and at 100°C (Figure 3.A.3a and b). Nevertheless, the radial distribution plots showed that in the outer layers of implants produced at 100°C, the protein concentration was lower than the average total concentration (Figure 3.A.3d). The lower surface concentration of proteins compared to implants prepared at 105°C (Figure 3.A.3c) correlated with the lower burst release of implants prepared at 100°C.

The coincidence of surface accumulation and loss of activity might suggest a relationship of protein distribution and its susceptibility to undergo rehydration-induced alterations (e.g., aggregation and/or structural changes via a higher local protein concentration). However, a more detailed study is required to address this question.

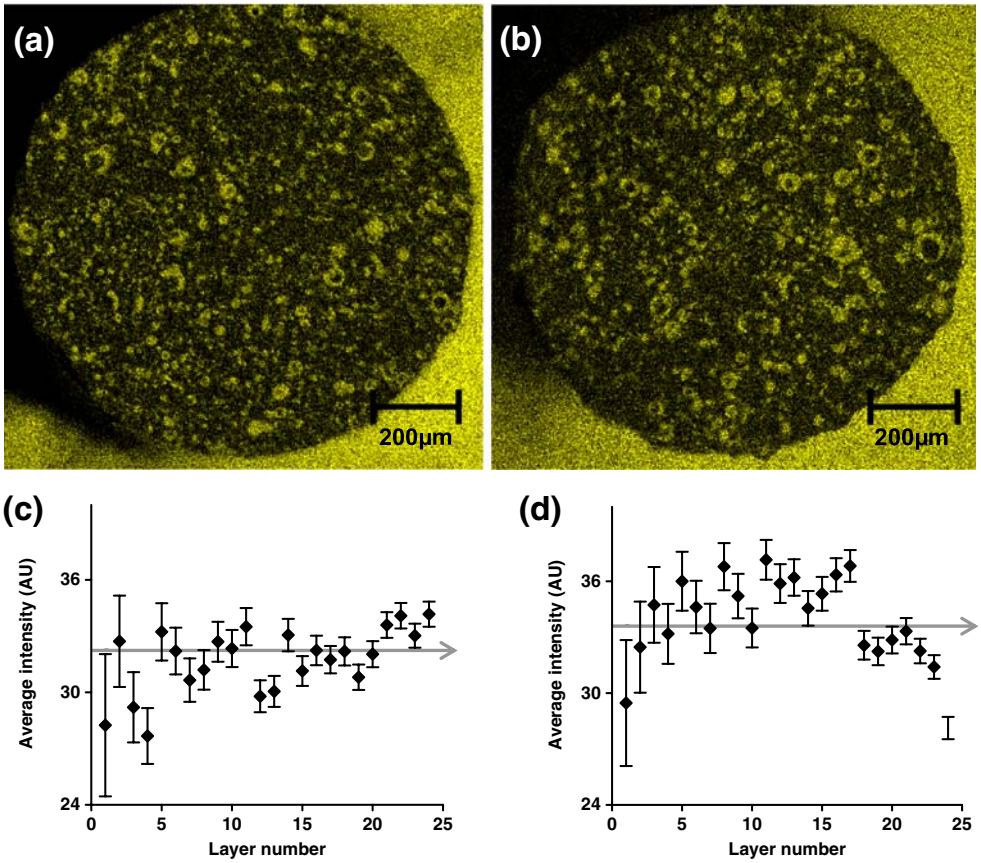

Figure 3.A.3 Lysozyme distribution illustrated with EDX-SEM elemental mapping of sulfur (yellow/white spots) on cross-section of 25% lysozyme containing PLGA implants prepared by hot-melt extrusion at (a) 105°C and (b) 100°C. The corresponding radial distribution plots, (c) 105°C and (d) 100°C, depict average of pixels intensity in each layer form inside (layer 1) to outside (layer 24). The gray arrows show the averaged total intensity. (see supplementary data for pixel analysis)

In addition to hydration, another stress factor which might cause incomplete protein release from PLGA controlled-release systems is the pH-drop inside the matrix due to trapped acidic polymer degradation products (van De Weert et al. 2000b). Exposure of the protein to the acidic environment during prolonged release can promote perturbation of protein structure and aggregation (J. Wang et al. 2004). Hence, lysozyme release from melt-extruded implants was followed and the achievable release patterns as well as the fate of the protein in the formulations were investigated.

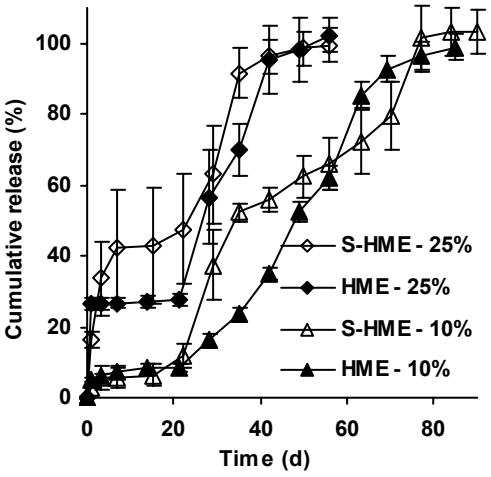

Figure 3.A.4 Comparison of the release from HME and S-HME PLGA implants. Legends indicate the method of preparation and lysozyme loading. Process temperature for HME was set on 90 and 100°C for 10% and 25% lysozyme-containing formulations, respectively. S-HME formulations were processed at 105°C

Hot-Melt vs. Syringe-Hot-Melt Extruded Implants

Melt-extrusion with a syringe-die device was used as a small-scale screening tool. The release of lysozyme from both types of melt-extruded PLGA implants showed a typical multiphasic pattern. The release profiles consisted of an initial burst followed by a period of negligible lysozyme release and an erosion-controlled release thereafter (Figure 3.A.4). The initial release at 10% lysozyme loading was similar for HME and S-HME implants. Increasing the lysozyme loading from 10% to 25% increased the initial release. The slightly higher burst with the 25% lysozyme-containing S-HME implant can be attributed to its lower density (higher porosity) compared to the HME matrix (1.15 gcm^{-3} vs. 1.45 gcm^{-3}). The lower variability in lysozyme release from HME compared to S-HME implants reflects a better homogeneity resulting from a better mixing of the formulations in the screw-type extruder than with the syringe-die device.

In all cases, the main protein fraction was released during the polymer erosion phase. The release rates of the S-HME and the HME implants were comparable resulting in a completion of the release from 25% lysozyme-loaded implants between 40 and 50 days (t90%). The residual amount was released between 50-60 days with full biological activity (96.7 ± 1.2%). However, deamidation, which can occur as a consequence of a pH decrease within the implant, can not be excluded based on these data. At low pHs,

deamidation has been shown to result in formation of protein derivatives with hyperactivity (Tomizawa et al. 1994).

Implants containing 10% lysozyme completed the release at about 80 days for both S-HME and HME. Thus, melt-extrusion with the syringe-die device was used as screening tool for HME formulations in the following studies.

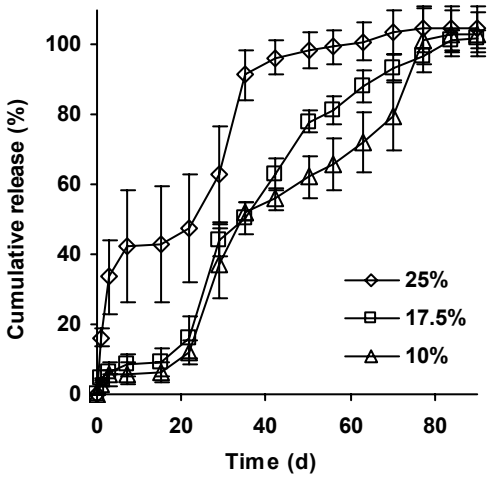

Figure 3.A.5 Effect of lysozyme loading on release from S-HME implants. Legends indicate lysozyme loadings

Effect of Drug Loading

The initial release phases were comparably small (<10%) for the 10% and 17.5% lysozyme-containing implants (Figure 3.A.5). Increasing the drug loading to 25%, however, increased the fraction released within the first week to about 40%. This suggests the drug percolation threshold being around 20%, above which some drug particles are in contact to form an interconnected network with access to the implant surface, resulting in drug release by diffusion through water-filled pores.

The erosion-controlled release phase for lysozyme particles not on the surface and not in contact with each other started at about day 20 for all three drug loadings. The release rates increased with increasing drug loading. As a result, release periods for 90% drug released of about 36, 63 and 73 days were obtained for the 25%, 17.5% and 10% drug loadings, respectively. The dependence of the release rate on drug loading might be

related to a fraction of remaining lysozyme particles in the matrix which can form an interconnected network. In addition, a higher lysozyme loading means a lesser PLGA content to be eroded. As well, a higher initial burst results in a more porous matrix which can accelerate both drug release and matrix erosion.

Effect of PEG Incorporation

PEGs with average molecular weights of 400 or 1500 Da and lysozyme were co-incorporated into S-HME implants in order to overcome the multiphasic release behavior of the protein implants through an increase of the diffusional drug release.

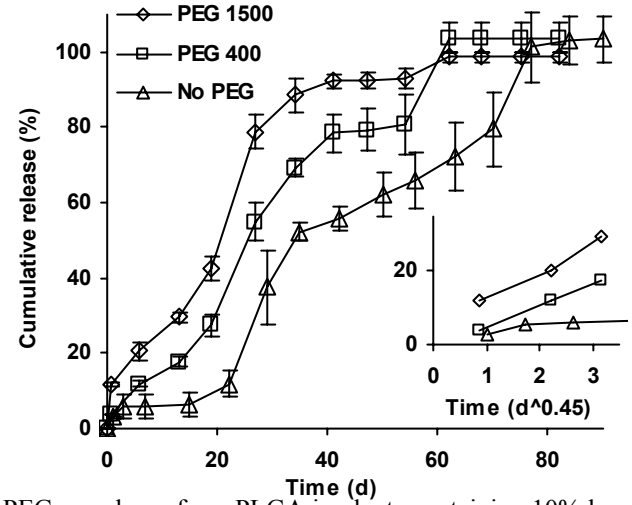

Figure 3.A.6 Effect of PEG on release from PLGA implants containing 10% lysozyme prepared with the syringe-die device with no PEG and 10% PEG 1500 or 400. The inserted graph shows lysozyme release in the first 2 weeks against time^0.45

Incorporation of 10% PEG (based on polymer) into 10% lysozyme-containing implants increased the rate and extent of lysozyme release during the first 14 days (Figure 3.A.6). The release approximated linear characteristics against time to the power of 0.45, which reflects a diffusion-controlled release for cylindrical shape matrices (Ritger & Peppas 1987) without an uncontrolled burst. The accelerating effect of PEG on the initial release of lysozyme correlated with an increased weight gain of the implants during incubation due to the uptake of release medium (Figure 3.A.7). Corresponding to the osmotic activity of drug and additive, the water uptake upon incorporation of PEG 400 was the highest, followed by PEG 1500 and the formulation without PEG. However, the release-

accelerating effect of PEG 400 was less pronounced compared to PEG 1500. This might be correlated to higher pore formation ability of PEG 1500.

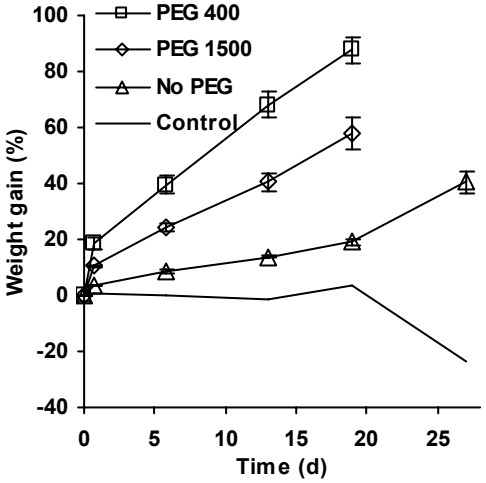

Figure 3.A.7 Weight gain of 10% lysozyme-loaded implants prepared with the syringe-die device with no PEG and 10% PEG 400 or 1500 compared to blank PLGA implant (control)

Similar differences in protein release between PEG 400 and PEG 1500 were reported (Taluja & Bae 2008; Bezemer et al. 2000). To better understand this difference and to obtain an optimized formulation with a high drug loading but low burst, the simultaneous effect of lysozyme and PEG loading on first day release was investigated. Implant formulations with loadings of 10%, 17.5% and 25% lysozyme (based on total) and 0%, 5% or 10 % PEG 400 or 1500 (based on polymer) were prepared. The initial lysozyme release of the PEG-free formulations increased with increasing drug loading (Figure 3.A.8a and 3.A.9a). The increase was pronounced when the drug loading was increased above 17.5%, which can be attributed to the drug percolation threshold.

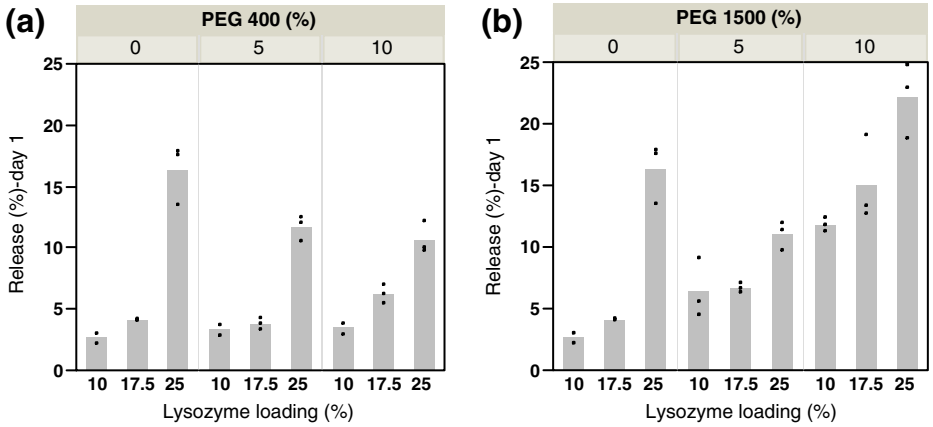

Figure 3.A.8 Lysozyme release on day 1 from S-HME implants vs. lysozyme loading at different concentration of (a) PEG 400- and (b) PEG 1500. Dots show n=3 data, if not overlapping, and bars show their average

Incorporation of PEG 400 reduced the effect of the protein loading on the initial release (Figure 3.A.8a). On the other hand, depiction of first day release vs. PEG 400 content showed that the effect of PEG on the release depended also on drug loading (Figure 3.A.9a). Below 22.5% drug loading, a slight increase in the release as a function of PEG concentration was noticed. This might be explained by a pore-formation through PEG. The amount of released protein from these formulations within the first day, however, was still low.

A more complex effect was seen above the percolation threshold of lysozyme. PEG appeared to decrease the initial burst release. The decrease might be explained by a PEG-induced viscosity increase which reduces diffusion through water-filled pores. When high levels of lysozyme (27.5%) and PEG (10%) were combined, however, the viscosity effect was diminished probably by an increased pore formation through further PEG addition. The result was a local minimum seen for the 27.5% lysozyme loading (Figure 3.A.9a).

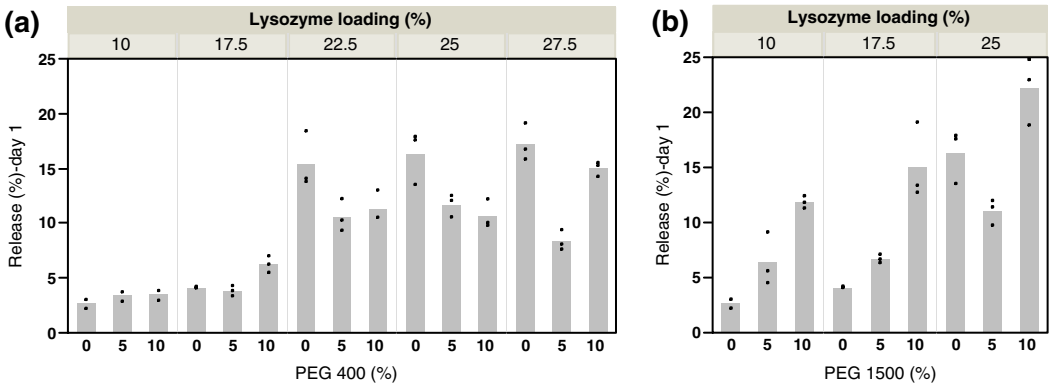

Figure 3.A.9 Lysozyme release on day 1 from S-HME implants vs. concentration of (a) PEG 400 and (b) PEG 1500 at different lysozyme loadings. Dots show n=3 data, if not overlapping, and bars show their average

The higher molecular weight PEG, however, increased the initial release at low lysozyme loadings (Figure 3.A.8b). Mass loss study showed nearly similar leaching of PEG 400 and 1500 from the matrix (6.3 ± 0.5 and 6.8 ± 1.2% respectively) within the first day. A stronger pore-formation effect through the formation of larger pores might have led to an acceleration of the initial lysozyme release with PEG 1500. Indeed, PEG 400 is a solvent for PLGA and mixes well with PLGA, while PEG 1500 is mixed in particulate form. Although being molten during melt extrusion, islands of PEG 1500 could potentially form in the PLGA matrix after extrusion since it does not dissolve PLGA.

As discussed here, the effect of PEG varied not only with size and concentration of PEG, but also with protein concentration. Lysozyme could be formulated with HME up to 20% loading without initial burst. Incorporation of 10% PEG 400 reduced the initial burst at 25% drug loading.

3.A.4 Conclusion

In this study, the feasibility of hot-melt extrusion for processing of proteins was examined with regard to the main challenges in the field, i.e. protein instability during manufacture and release, as well as the release incompleteness.

Nearly complete recovery of active lysozyme as a model protein illustrated that the melt extrusion process did not damage the protein integrity.

Melt-extrusion with syringe could be applied as a screening tool for optimizing hot-melt extrusion formulations. Lysozyme was completely released from all formulations whereby the initial release as well as release rate were controlled by lysozyme loading and additives. Drug release was also dependent on matrix properties including matrix density and drug distribution. Nearly complete enzymatic activity was obtained with the last fraction of released lysozyme from HME implants.

In summary, hot-melt extrusion is a promising method for the effective delivery of protein therapeutics because of its relatively simple, single step formulation process and good protein stability.

3.A.5 Supplementary Data

Analysis of Pixel Intensities in EDX Pictures

The following MATLAB® function computes the radial distribution of proteins in the EDX pictures of (circular-shaped) implant's cross-section. This is performed by considering a number of concentric layers in the circle and computing the average intensities of pixels in each layer (in a grayscale picture).

First, a circle was fitted to each image and the information of this circle, together with the original figure were used as the input of the pixel analysis function (Figure 3.A.10). This function has five input parameters: (1) the image file name, (2 and 3) x and y coordinates of the circle offset, (4) r, the radius of the circle and (5) l, the number of levels desired for the circle.

The function prints two vectors as the output. The first vector is the average pixel intensity in each layer (ordered from the center to the perimeter). The second vector is the 95% confidence interval of the intensity distributions in each layer (with the assumption that the data are distributed as a Student's t-distribution).

Parameters of fig4a: x = 75, y = 20, r = 174
Parameter of fig4b: x = 85, y = 29, r = 168
L = 24

****

```
function pixel (img, x, y, r, l)
   % img = image file name
   % x, y = circle offset
   % r = circle radius
   % l = number of layers
   I = double(imread(img));
   if (ndims(I)==3)
      grey = (I(:,:,1)+I(:,:,2)+I(:,:,3))/3;
   else
      grey = I;
   end
   imwrite( uint8(grey), strcat(img, 'grey.bmp'));
   s = size(grey)
   v = zeros(l, 3);
   for i=1:s(1)
      for j= 1:s(2)
         c = sqrt((i-y-r)*(i-y-r)+(j-x-r)*(j-x-r))*l/r;
         c = floor(c);
         if (c<l)
            v(c+1, 1)=v(c+1, 1)+grey(i, j);
            v(c+1, 2)=v(c+1, 2)+1;
         end
      end
   end
   layer_average = v(:, 1)./v(:, 2);
   confidence_interval = zeros(l, 2);
   for k=1:l
      u = zeros(v(k, 2),1);
      e = 1;
      for i=1:s(1)
         for j= 1:s(2)
            c = sqrt((i-y-r)*(i-y-r)+(j-x-r)*(j-x-r))*l/r;
            c = floor(c);
            if (c+1==k)
               u(e) = grey(i, j);
               e = e+1;
            end
         end
      end
      [h, p, ci, stats]=ttest(u);
      confidence_interval(k, :) = ci;
   end

   layer_average
   confidence_interval
```

****

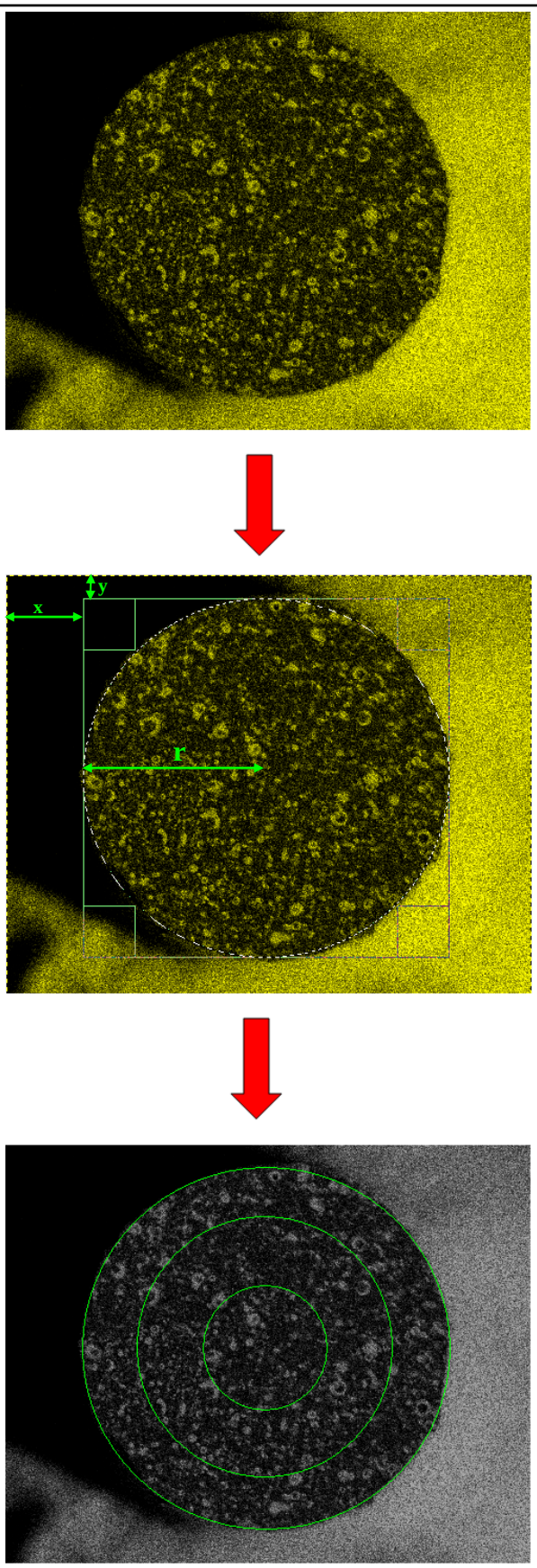

Figure 3.A.10 Schematic representation of pixel analysis procedure

Effect of Heating-Rate on Melting Temperature Measured by DSC

Increasing the heating rate during DSC scan had minor effect on the measured melting point. By increasing from 5 to 20 K/min, Tm increased from 200°C to 205°C (Figure 3.A.11).

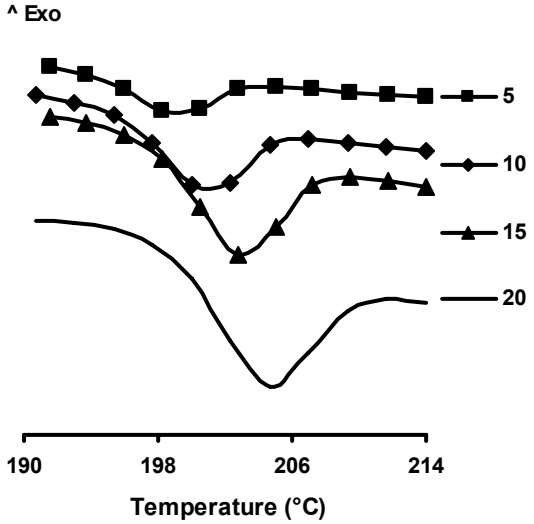

Figure 3.A.11 Effect of heating rate (K/min) on lysozyme T_m measured by DSC

Effect of Protein Detection Method on the Estimated Protein Recovery

In addition to BCA assay, lysozyme recovery upon hydration (1 day release) was assessed with two other methods: Coomassie assay and UV absorbance at 280 nm. Before spectroscopic analysis, samples were heated in 3 M guanidine hydrochloride to quantify the total lysozyme content as a control. This treatment can disrupt all non-covalent interaction and make the assay independent of protein structure. Similar data was obtained from BCA and spectroscopic measurements (Table 3.A.2) which revealed appropriateness of BCA assay for total protein quantification. Coomassie assay resulted in significantly lower values. Consequently, the estimated total recovery by Coomassie was as low as 63% (Table 3.A.2). This can be due to presence of some unknown interferences or might illustrate involvement of lysozyme basic groups in other interactions which inhibited them from interaction with Coomassie. The impact of detection method, as briefly examined here, suggests the necessity of a revision in reported cases of incomplete release. Indeed, there is a possibility that proteins are released but unsuitability of detection method for the relevant system, leaded to evaluated incomplete release.

Table 3.A.2 Effect of detection method on protein recovery from 25% lysozyme loaded HME implants

	Spectroscopy	BCA	Coomassie
1 day release (%)	41.9 ± 3.8	41.9 ± 2.9	14.9 ± 1.3
Total recovery (%)	100.1 ± 1.5	100.0 ± 2.9	62.9 ± 2.1

B

Protein Release from Poly(lactide-co-glycolide) Implants Prepared by Hot Melt Extrusion: Thioester Formation as Reason for Incomplete Release

The purpose of this study was to characterize bovine serum albumin release from PLGA-based implants prepared by hot melt extrusion with special emphasis on the reason for incomplete release and ways to optimize the release accordingly.

3.B.1 Background

Growing attention has been paid to the parenteral delivery of peptides and proteins with biodegradable injectable delivery systems in order to facilitate their controlled delivery and to enhance their therapeutic efficiency (Giteau et al. 2008a).

PLGA is the most popular biodegradable polymer. This can be ascribed in part to the approval of several PLGA-based drug-delivery devices by the FDA for human use. Most marketed products are extended release formulations of peptide hormone analogues (GnRh-agonists and ocreotide), whereas very few proteins have shown their suitability for a controlled delivery from PLGA-based devices (J. H. Kim et al. 2005). Difficulties associated with the encapsulation process of proteins are typically related to their large molecular weight, high water solubility, and chemical and physical instabilities (Crotts & Park 1998). Proteins can undergo degradation and/or aggregation during the fabrication of PLGA-based formulations, which often results in unpredictable release profiles, characterized by a burst effect and incomplete release (Panyam et al. 2003).

Several stress factors can compromise the integrity of a protein during preparation, storage and release from biodegradable delivery systems. Depending on the encapsulation method, exposure of proteins to water/organic solvent interfaces, protein-PLGA interaction, shear and/or drying are the major destabilizing factors during preparation (van De Weert et al. 2000). Hot melt extrusion (HME) offers a solvent-free manufacturing process to prepare biodegradable devices. Many stress factors can be avoided. In addition, deleterious effects on the protein associated with residual solvents will be excluded. Nonetheless, elevated temperatures in HME process have been reported to initiate formation of peptide degradation products during manufacturing of PLA-based implants (Rothen-Weinhold et al. 2000). Proteins, however, are in the solid-state in the typical process temperature range for PLGA (80 – 120°C) and are thus expected to have a higher thermal stability. Hen egg white lysozyme, an example of a rather stable protein, was not adversely affected during an HME process run at 105°C (Ghalanbor et al. 2010). During storage, moisture or dehydration/desolvation can initiate proteins instability (Prestrelski et al. 1995). Hydration by the release medium can increase structural mobility and lead to unfolding or misfolding of proteins (Hill et al. 2005). The acidification within degrading

PLGA matrices (Estey et al. 2006) as well as non-covalent protein-PLGA interactions (hydrophobic and/or electrostatic) were supposed to be the major causes of incomplete protein release in vitro (van De Weert et al. 2000a).

Bovine serum albumin is a relatively large (66.4 kDa) globular protein which is highly prone to aggregation (Militello et al. 2003). It can form water-insoluble aggregates upon exposure to moisture (Liu et al. 1991) or during the release from PLGA nano- or microparticles or implants (Panyam et al. 2003; M. S. Kim et al. 2005; Zhu et al. 2000). As a result, BSA release from PLGA-based delivery systems is often biphasic, with an initial burst followed by a slow and incomplete release. The possibility of chemical reactions with degradation products of PLGA, which has been reported for peptides (Murty et al. 2005), has also to be considered as the reason for the incomplete release besides protein-protein interactions. PLGA oligomers caused acylation of the peptides. Nucleophilic functional groups including primary amines (Lucke et al. 2002) and hydroxyl groups (D. H. Na et al. 2003) were suggested as the potential reactive sites.

The purpose of this study was to optimize BSA release from PLGA-based HME implants with regard to release rate and completeness and to elucidate the reason for the incomplete release of protein-loaded PLGA matrices.

3.B.2 BSA Stability during Hot-Melt Extrusion

Elevated temperature in combination with shear forces are the potential stress factors during hot-melt extrusion for protein structure and integrity. Aggregation is the main mechanism of BSA instability during encapsulation in PLGA delivery systems (Taluja et al. 2007). Therefore, BSA was examined for aggregation after extrusion at 105°C.
ATR-FTIR spectra of 10% BSA-loaded PLGA-based implants before (physical mixture) and after hot melt extrusion showed typical bands for PLGA, protein amide I and II bands at 1750, 1650 and 1530 cm^{-1},respectively (Figure 3.B.1) (van de Weert et al. 2000c). Intermolecular ß-sheets of non-covalent BSA aggregates or unfolded protein can be identified by an additional band at 1624 cm^{-1} and/or a shift of the amide I band in FTIR spectra to lower wavenumbers, respectively (Tantipolphan et al. 2008). The absence of

these features in the spectra indicated that the integrity of BSA was not affected by the HME process.

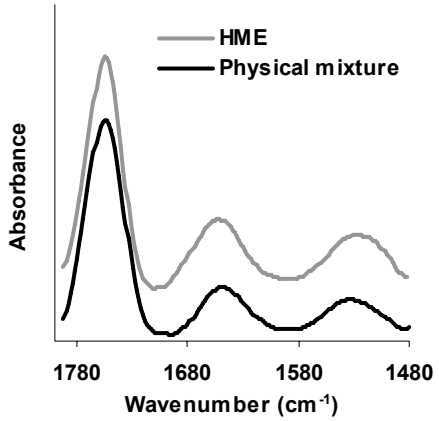

Figure 3.B.1 ATR-FTIR spectra of BSA-PLGA physical mixture and ground PLGA implant (25% BSA content). Peaks at 1750, 1650 and 1530 cm^{-1} represent PLGA, protein amide I and II bonds, respectively

Differential scanning calorimetry revealed a similar denaturation temperature (T_m) of 220°C for unprocessed and HME-processed BSA, which supported the preservation of protein structure (Figure 3.B.2).

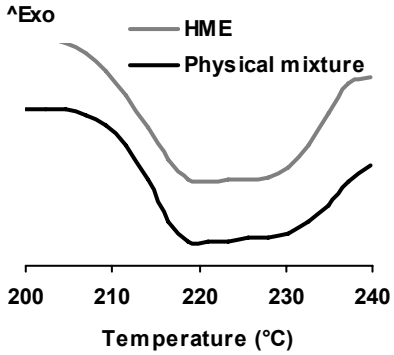

Figure 3.B.2 DSC thermograms of BSA-PLGA physical mixture and ground PLGA implant (25% BSA content)

The absence of insoluble protein aggregates was indicated by a complete recovery of BSA upon extraction of the implants. The extracted proteins were analyzed by MALDI-MS, which facilitates detection of protein derivatives in the nano-molar range (Kukhtina et al. 2000). In accordance with FTIR and recovery results, the MALDI-MS spectrum of

BSA extracted from the implant was similar to the BSA control (Figure 3.B.3). Hence, no degradation or peak shifts or broadenings became apparent.

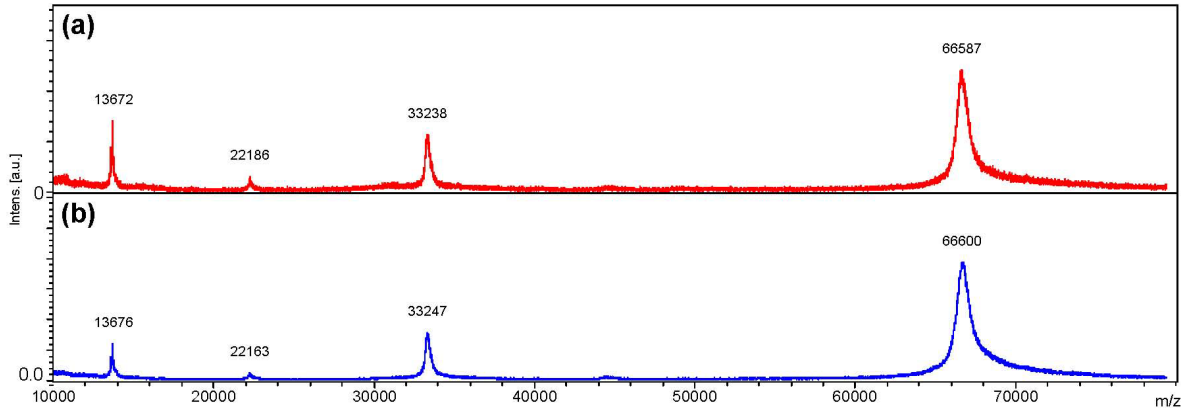

Figure 3.B.3 Mass spectra of (a) pure BSA powder compared to (b) extracted BSA from HME implant (25% BSA content)

Protein stability during hot melt extrusion was also confirmed for two other model proteins cytochrome C and ovalbumin with FTIR and DSC (data not shown, similar to figures 3.B.1 and 3.B.2). These data, in addition to previous results on lysozyme (Ghalanbor et al. 2010), suggest that hot melt extrusion could be used to incorporate proteins into PLGA implants without affecting their structural integrity.

3.B.3 BSA Release

Effect of BSA Particle Size on Initial Burst Release

BSA release from hot melt extruded implants containing 10 or 25% protein showed burst releases of 12% and 72%, respectively (Figure 3.B.4), which were much higher compared to previous results on lysozyme (Ghalanbor et al. 2010). The volume mean diameter of the manually ground material was 46.7 µm. Large protein particles in implant cross-sections were confirmed by elemental mapping (Figure 3.B.5a). A reduction of the BSA particle size by ball-milling to a volume mean diameter of 17.7 µm resulted in a more homogenous distribution of protein in the implant (Figure 3.B.b). As a consequence of ball-milling, the initial burst release from both 10% and 25% protein-loaded implants decreased to 1.5% and 5.6% respectively (Figure 3.B.4). Ball-milling did not affect the

structure of BSA (DSC and FTIR data not shown). The reduced burst release was therefore attributed to a better entrapment of the smaller BSA particles into the implants.

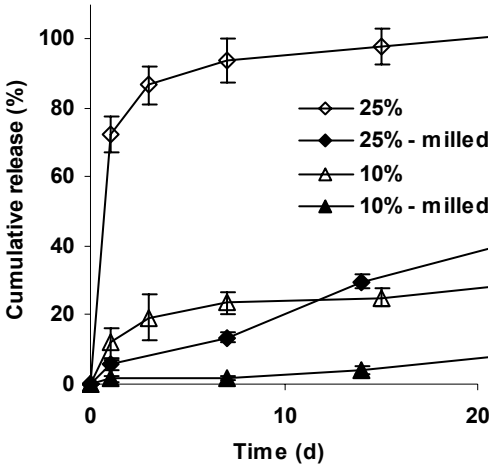

Figure 3.B.4 BSA release of PLGA implants as a function of protein content and milling

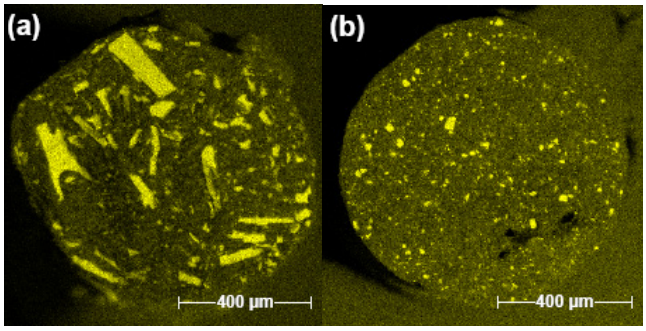

Figure 3.B.5 BSA distribution on cross-sections of 10% BSA-containing PLGA implants illustrated with EDX-SEM elemental mapping of sulfur (yellow/white spots) (a) before and (b) after ball-milling

Effect of BSA Loading on Release

The first release phase up to about 20 days was not affected much by BSA loading up to 20% but increased significantly by increasing the loading to 25% and 30% BSA (Figure 3.B.6). This was attributed to exceeding the percolation threshold of the protein in the PLGA matrix. This first release phase, however, was not a burst, which mostly ends within one day (Luan & Bodmeier 2006), but controlled release over about 20 days. This might be due to a slow but continuous diffusion process, since the implant erosion started afterwards (see chapter 3.C).

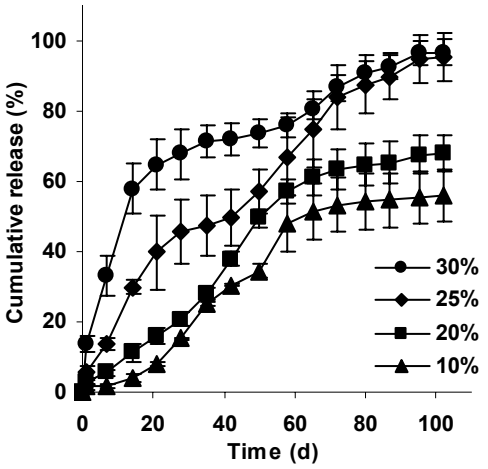

Figure 3.B.6 Effect of BSA loading on the release from PLGA implants

The BSA loading also affected the release completeness. The total protein release did not exceed 70% at loadings below the percolation threshold (10% and 20%). Here, the release was mainly attributable to PLGA erosion starting after day 20. The release of the 10 and 20% BSA-loaded implants plateaued at around day 70, which was attributed to a lower porosity of the non-percolating samples upon release. Interestingly, a part of the implant was still present in the release medium and did not dissolve up to day 180, when the experiment was stopped. Analysis of the insoluble residual implant material of the 20% BSA-loaded implants after 110 days release with ATR-FTIR revealed the presence of both PLGA and BSA (Figure 3.B.7), with a PLGA:BSA mass ratio of about 1:1. To test for protein aggregation or adsorption, the residual implant was incubated in 6 M GnHCl, which should dissociate non-covalent bonds (Park et al. 1998). However, the additional BSA release was not increased by more than 2% after 2 weeks of incubation in the denaturing solution. Non-covalent aggregation was thus not responsible for the residual mass and thus the incomplete release.

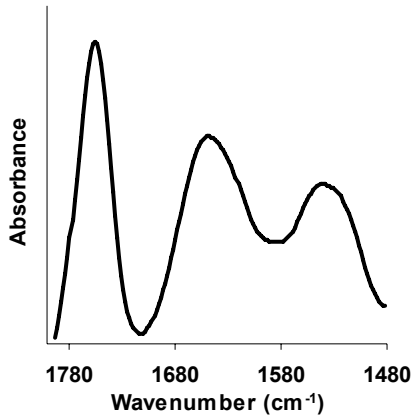

Figure 3.B.7 ATR-FTIR spectra of 20% BSA-loaded implants after release (110 days). Peaks represent PLGA, protein amide I and II bonds at 1750, 1650 and 1530 cm^{-1}, respectively

The presence of PLGA in the residual mass was unexpected, since PLGA should be already degraded to water soluble oligomers within 50-60 days (Sandor et al. 2001). Addition of sodium hydroxide (1 M) to the release medium dissolved the residual mass completely in less than 30 min and facilitated the total recovery of BSA. Sodium hydroxide leads to the alkaline hydrolysis of esters (e.g. within PLGA or possibly between PLGA and BSA), whereas amides are known to be stable under alkaline conditions.

The addition of dithiothreitol (DTT, 0.01 M) resulted in dissolution of the insoluble residual mass. In contrast to NaOH, it did not dissolve PLGA in a control experiment. This indicated that the residual implant mass did not represent insoluble PLGA entrapping residual BSA but rather an insoluble protein-PLGA mass. DTT is capable of cleaving disulfide bridges and thioester bonds (Fenton & Fahey 1986), which BSA could have formed with its reactive free thiol group (Cys34) (Valdebenito et al. 2010; Pedersen & Jacobsen 1980). Thus, either intermolecular BSA-BSA interactions or BSA-polymer reaction products could explain the residual insoluble mass. A possibility to differentiate between both reaction products is the addition of hydroxylamine, which can only hydrolyze thioesters but not disulfide bridges (Fenton & Fahey 1986). Addition of hydroxylamine (0.2 M, pH 7) dissolved the insoluble implant mass completely. This suggested the formation of thioester bonds between BSA and PLGA.

In accordance with previous results (Zhang et al. 1993), an increase of the total release with increasing drug loadings was attributed to a higher porosity of the percolating (> 25% BSA) compared to non-percolating formulations (10 and 20% BSA). To test for an effect of matrix porosity on the release completeness, 20% BSA-loaded implants with a higher porosity were prepared. Therefore, implants where prepared with PLGA containing 8% ethanol which resulted in implants with almost half the density compared to the original implants (0.65 vs. 1.15 g/cm^{-3}), due to the evaporation of ethanol during HME. Implants prepared with ethanol showed an increased diffusional release (Figure 3.B.8) and complete BSA release without leaving any residual insoluble material. Thus, the formation of thioesters decreased upon an acceleration of BSA release due to the faster diffusion through a more porous implant. This could be explained with a minimal contact between protein and acidic PLGA degradation products.

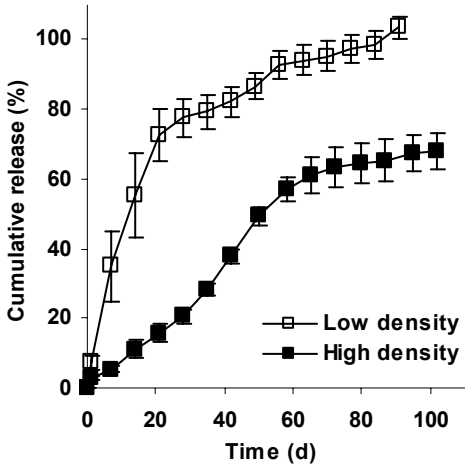

Figure 3.B.8 Effect of implant density on BSA release from 20%-loaded PLGA implants

As suggested here, PLGA-protein interaction, mediated by free cysteine residues of proteins, can be one of the reasons for incomplete release of proteins from PLGA delivery systems. Therefore, it can be hypothesized that modification of cysteine residues provides a better chance for release completeness. Cysteine can be chemically capped e.g. by site-specific PEGylation of cysteine residues (Roberts 2002). PEG-interferon-α is a commercial example with improved pharmacokinetic profile. As well, Cysteine can be replaced by other amino acids like serine as been done for Proleukin (Chiron Corp), which is a commercially available analogue of human interleukin-2 with improved

therapeutic effect (Frokjaer & Otzen 2005). However complete and extended release could also be achieved when diffusion exceeded PLGA degradation rate, e.g. with optimizing matrix properties. This might be a more feasible strategy to circumvent some of the stability issues related to PLGA oligomers and to avoid complicated chemical modifications.

3.B.4 Conclusion

BSA was incorporated into PLGA implants using hot-melt extrusion without compromising its structural integrity or impurity formation.

PLGA implants with up to 25% BSA loading could be formulated without initial burst release. The total protein release varied from 60 – 97% and increased with increasing loading. Incomplete release was accompanied by presence of a remaining insoluble PLGA- and BSA-containing mass, which could be identified as a covalent adduct of BSA and PLGA by thioester linkages. An accelerated BSA release achieved through an increase in implant porosity led to complete protein release. This was attributed to a minimized contact of protein and reactive PLGA degradation products.

C

Interdependency of Protein-Release Completeness and Polymer Degradation in PLGA-Based Implants

The purpose of this study was to increase the completeness of BSA release from PLGA implants.

3.C.1 Background

Biodegradable implants have been investigated as potential parenteral delivery systems for the extended release of peptide and protein therapeutics (Santoveña et al. 2010; Rothen-Weinhold et al. 2000). While low-molecular-weight drugs and peptides have been successfully incorporated into biodegradable poly(lactide-co-glycolide) (PLGA) matrices (Shameem et al. 1999), larger protein drugs have been more problematic due to the susceptibility of their structure and hence their functionality (Ghassemi et al. 2009).

Protein release from PLGA devices is often characterized by an initial diffusion-controlled burst release followed by slow and incomplete release (Ghassemi et al. 2009) despite extensive degradation of the polymer (Giteau et al. 2008a). Incomplete protein release is often attributed to protein instability within the PLGA devices. One of the sources of protein denaturation and aggregation during release is the exposure of proteins to the acidic microclimate, which develops due to the formation of acidic oligomers during the degradation of PLGA (Estey et al. 2006). The formation of insoluble non-covalent protein aggregates was previously attributed to the acidification within PLGA matrices (Panyam et al. 2003; G. Zhu et al. 2000). The co-incorporation of an antacid was therefore suggested as a stabilization approach (G. Zhu et al. 2000). Although the suppression of the non-covalent aggregation increased the diffusional release within the first 7 days, the total protein release remained incomplete. The short release period, however, excluded effects of acidic degradation products on the release of the protein, since PLGA erosion is expected to start afterwards (Brodbeck et al. 1999). Mechanistic evaluations of a relationship between PLGA oligomer formation and protein release would thus require longer observation periods.

Incomplete release can also be the result of hydrophobic or ionic interactions between entrapped proteins and PLGA, resulting in adsorption of the protein to the carrier (Bilati et al. 2005) Adsorbed and partially unfolded protein can subsequently form multiple layers of insoluble, aggregated protein (Vörös 2004). Besides physical interactions, close contact between protein molecules or protein and PLGA molecules can result in chemical reactions. BSA, for example, formed insoluble covalent PLGA-BSA adducts via thioester-bonds upon prolonged incubation under release conditions (see chapter 3.B).

The presence of PLGA in the residual implants after 180 day release, however, was unexpected, considering the degradation rate of the polyester (Tracy et al. 1999; Sandor et al. 2001).

PLGA co-polymers degrade via hydrolysis of the ester bonds. Formation of carboxylic acids during degradation of the polyesters can accelerate the hydrolysis of other ester bonds, a process called autocatalysis. When molecular weight of PLGA oligomers approach the solubility boundary (~ 1000 g/mol (Körber 2010)), they can diffuse out of the polymeric matrix through a degradation- and solubility-controlled process (Körber 2010). Consequently, mass-loss of the matrix (erosion) starts which undergoes through the balk (van De Weert et al. 2000b).

In order for the drug to affect polymer hydrolysis it needs to partition into the polymer phase (Wischke & Schwendeman 2008). In case of basic compounds degradation rate can be reduced via ionic interaction of the drug with PLGA end-groups. Additives and drugs can also change the matrix properties, e.g. by increasing matrix hydrophilicity or porosity and thereby enhancing escape of degradation products and reducing autocatalysis (Jonnalagadda & Robinson 2004). Proteins generally can not partition into hydrophobic polymer phases (Kang & Schwendeman 2007). However, how proteins may affect PLGA degradation has not been well studied.

Bovine serum albumin (BSA) is a relatively large model protein (66.4 kDa) which is highly aggregation-prone (Militello et al. 2003), particularly upon exposure to moisture (Liu et al. 1991). Solvent-free processing using hot-melt extrusion (HME) provided a tool for the solvent-/moisture-free incorporation of BSA into PLGA implants (see chapter 3.B). The implants exhibited multiphasic release profiles, which is typical for PLGA delivery systems (Santander-Ortega et al. 2009). The initial diffusion-controlled release was followed by an erosion-controlled release which was incomplete (see chapter 3.B).

The degradation and erosion of PLGA as well as the release of BSA were characterized in order to understand and to find formulation approaches facilitating complete release of BSA from PLGA implants.

3.C.2 BSA Release

Drug release profiles of PLGA implants with 10% and 25% BSA loading were characterized by a low initial release of less than 10% followed by a slow release phase of about 4 weeks before an erosion-controlled release phase started (Figure 3.C.1). BSA release leveled off around day 80, up to which only one third (10% BSA loading) or half (25% BSA loading) of the protein content was released. A residual insoluble mass remained intact over 180 days. It was recently shown, that the residual implant consisted of insoluble thioesters of BSA and soluble PLGA-oligomers (see chapter 3.B). Since PLGA should be degraded within 50-60 days (Sandor et al. 2001) it was hypothesized that degradation and/or erosion rate of the polymer was reduced in presence of BSA.

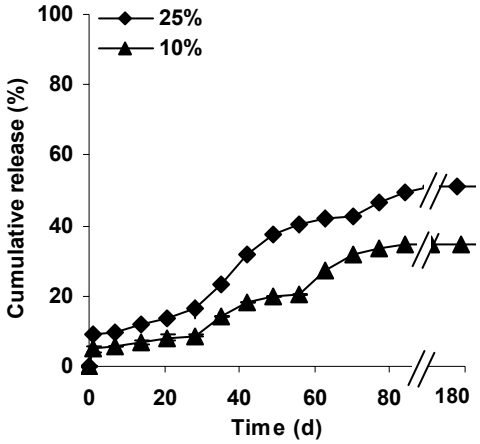

Figure 3.C.1 Effect of BSA content on the release of PLGA implants in PBS pH 7.4

3.C.3 Effect of BSA on PLGA Degradation

Hydrolysis of end-capped PLGA usually starts after as induction period (Dunne 2000) which is the time before formation of enough free carboxyl groups to start autocatalysis and the accelerated degradation phase. In case of RG 502 this period was about 10-14 days followed by a pseudo-first order reaction kinetic where the peak of the lognormal molecular weight distribution of PLGA (log Mp) decreased linearly with incubation time (Figure 3.C.2). The peak molecular weight approached the molecular weight of soluble PLGA (log Mp ~3) within 35 days for BSA-free implants.

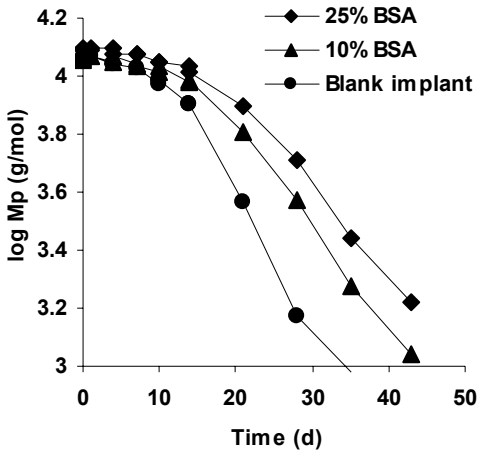

Figure 3.C.2 Semi-log plot of the peak molecular weight of PLGA implants during degradation in PBS pH 7.4 as a function of BSA content

Incorporation of BSA decreased the degradation rate notably (Figure 3.C.2) which was attributed to the reduced autocatalysis effect as a result of earlier outflux of acidic degradation products (Figure 3.C.3). The slower degradation of the BSA-containing implants was accompanied by a higher initial medium uptake (Figure 3.C.4). In addition protein release can increase porosity of the implants which induced outflux of acidic degradation products and thus reduction of pH in the release medium (Figure 3.C.3).

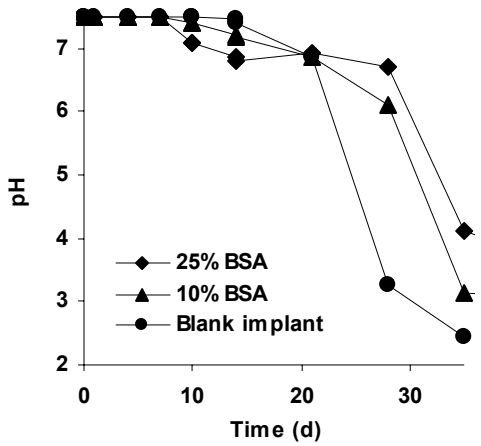

Figure 3.C.3 pH change in the release medium during degradation in PBS pH 7.4 as a function of BSA content

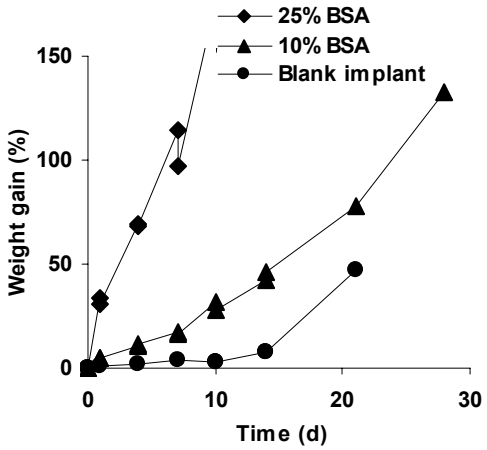

Figure 3.C.4 Weight gain of PLGA implants in PBS pH 7.4 as a function of BSA content

3.C.4 PLGA Erosion

Erosion of the BSA-free implants started after 20 days and was completed within about 50 days (Figure 3.C.5a) without leaving any residual material. In BSA-containing implants mass loss started earlier after 14 days, as discussed above. However, the accelerated erosional mass loss started later after 28 days (Figure 3.C.3 and 3.C.5a). The slower erosion rate of BSA-containing implants could be merely due to their slower degradation rate.

The erosional mass loss of the 10% and 25% BSA-loaded implants stopped between days 50 – 60, leaving behind an implant mass, which was insoluble in water and in the polymer solvent THF. The implants, however, had lost only 60% (10% BSA-loading) and 80% (25% BSA-loading) of the polymer content up to this point (Figure 3.C.5a). The incomplete polymer erosion was in agreement with the formation of insoluble covalent adducts of PLGA-BSA (see chapter 3.B), which were not soluble in THF. The extent of PLGA mass-loss of 10% and 25% BSA-loaded implants was correlated with the total releasable protein fraction form these implants.

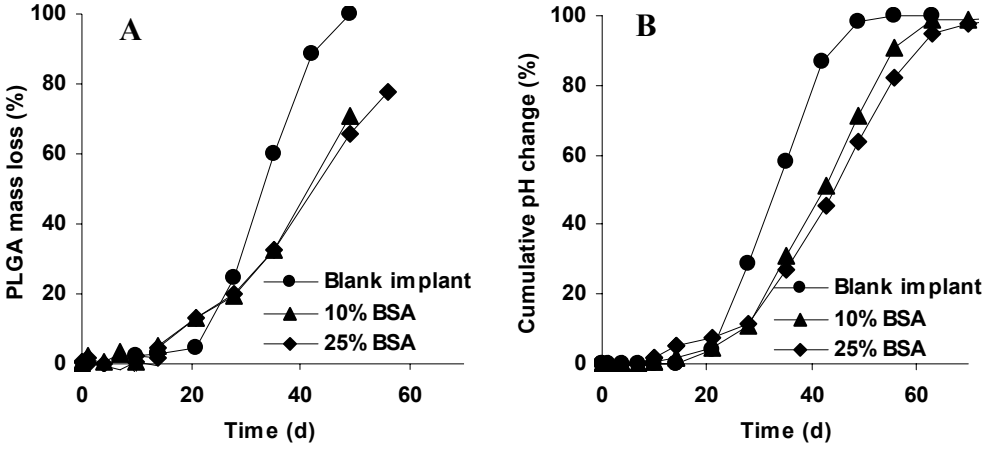

Figure 3.C.5 Effect of BSA loading on (a) the polymer mass-loss of degrading PLGA implants in PBS pH 7.4 and (b) cumulative medium-pH changes

PLGA mass loss was accompanied with an acidification in the release medium due to the release of acidic oligomers. Accumulating the pH changes over time resulted in sigmoidal profiles (Figure 3.C.5b), which superimposed the PLGA mass loss profiles for the major part of the erosion period (Figure 3.C.6). Sigmoidal erosion patterns are typically seen for PLGA matrices, since they are the consequence of the degradation time-dependent shift of the molecular weight distribution towards the boundary, where PLGA oligomers become water soluble (1000 g/mol) (Körber 2010).

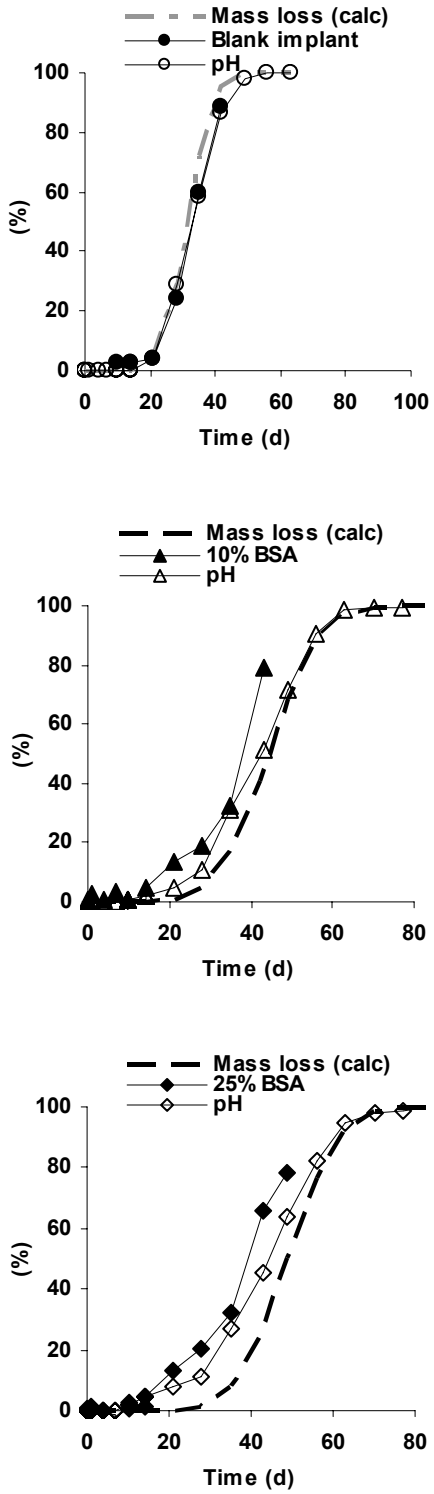

Figure 3.C.6 Overlaying of medium pH change on the actual and calculated mass-loss profiles of blank, 10% and 25% BSA-loaded implants

Based on the polymer degradation kinetics (Figure 3.C.2), the formation of soluble oligomers was calculated for each implant, using a recently reported model which estimates PLGA erosion based on the measured degradation rate (Körber 2010). The formation of soluble oligomers was in excellent agreement with the erosional mass loss of the blank implants (Figure 3.C.6), which indicated that PLGA erosion was only controlled by the formation of soluble oligomers and hence the degradation process. The actual mass loss of the 10% and 25% BSA-loaded implants, however, were ahead of the calculated profile (Figure 3.C.6). This indicated that in presence of BSA the release of degradation products was enhanced at early time points.

3.C.5 Improvement of Protein Release

The contact between BSA and PLGA should be minimized in order to decrease their reaction with each other and formation of insoluble adducts and thus to enhance completeness of the release. Such contact can potentially occur both during the induction period and during erosion. To test whether the induction period would affect the release completeness, PLGA was pre-degraded for 2 weeks (Mp ~ 8700 g/mol) and used to prepare the HME implants.

BSA release from these implants was slightly faster when compared to the original implants (Figure 3.C.7a). The release profiles indicated a difference between the onsets of the erosion controlled release phase of two weeks, which reflected the pre-degradation period. Accordingly, the pH profiles of the exchanged release media revealed a two weeks faster erosion onset using the pre-degraded PLGA. In contrast to the original implants, the pH profiles were independent of the BSA-loading (Figure 3.C.7b), which supported that BSA did not affect the degradation rate directly, as discussed above.

However, the shortening of the contact time between BSA and PLGA, by skipping the induction period, did not improve the release completeness of the implants (Figure 3.C.7a). It was therefore concluded that the contact time of BSA to soluble PLGA degradation products during the erosion phase should be reduced to decrease their interaction and hence increase the completeness of the protein release.

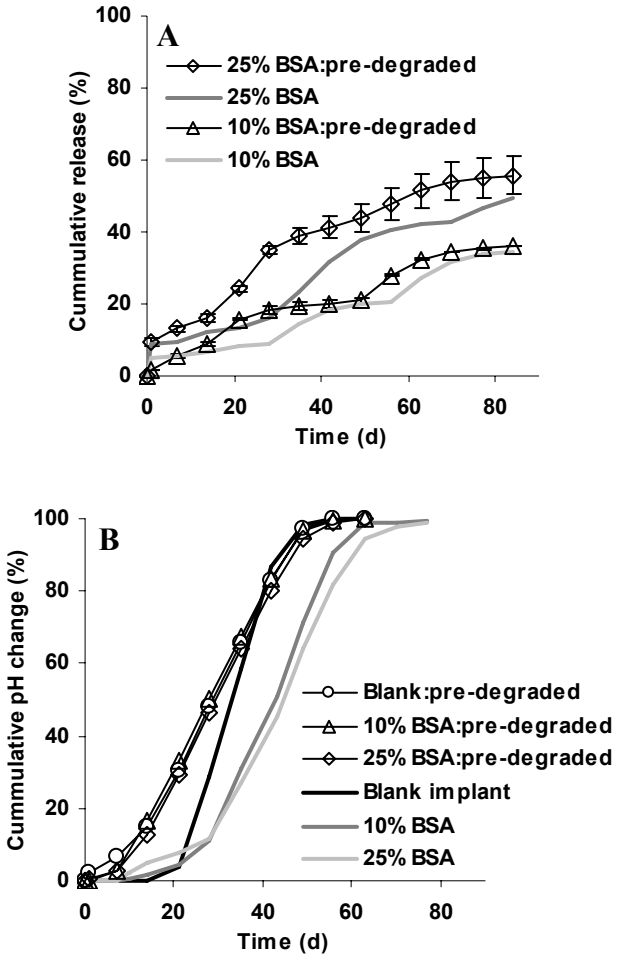

Figure 3.C.7 Effect of BSA loading on (a) the release and (b) cumulative pH change of 14 days pre-degraded PLGA implants compared to the original implants in PBS pH 7.4

The initial size of incorporated BSA particles affected its initial release from hot-melt extruded implants (see chapter 3.B). PLGA implants loaded with larger BSA particles (mean size: 48 μm vs. 18 μm) showed a high initial burst (75%) but complete release within 42 days (Figure 3.C.8). Correspondingly, the PLGA matrix dissolved completely within 70 days, as estimated by the erosion model (Figure 3.C.6, 25% BSA). Although characterized by an undesirable high burst, complete BSA release and erosion was achievable by reducing the exposure of protein to the reactive oligomers present during PLGA erosion.

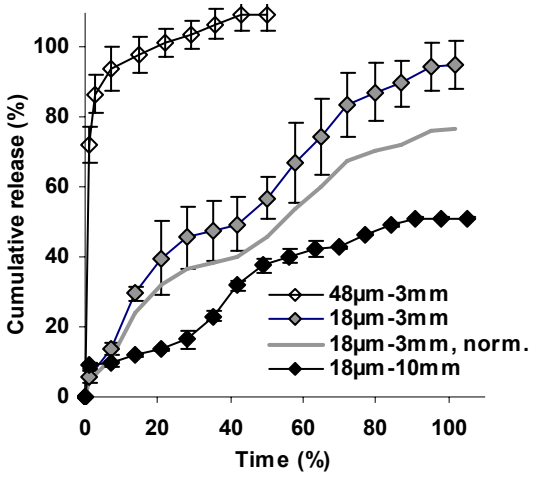

Figure 3.C.8 Effect of initial protein particle size (µM) and implant length (mm) on BSA release from 25% protein-loaded implants. The gray line shows the normalized release of 18µm-3mm implants for the ratio of surface area / mass compared to 10 mm ones.

A reduction of the implant size from 10 mm to 3 mm length increased the diffusional release without a high initial burst (Figure 3.C.8). This was partly due to the higher surface area / mass ratio of the small implant (1.24 fold). However, normalization of the release to this ratio did not decrease the release of 3 mm implants to that of 10 mm ones (Figure 3.C.7). This suggested that the release from its cut ends of the implant was higher than from its longitudinal surface.

Table 3.C.1 Effect of implant size on the fractions of protein released from 25% BSA-loaded PLGA implants during the first 20 days (diffusion) and from day 20 to the end of release (erosion)

Implant size (mm)	3	7	10
Diffusional release	39.8 ± 10.6	27.7 ± 2.19	13.6 ± 0.2
Erosional release	55.3	43.2	37.5
Unrecovered fraction	4.9 ± 6.7	29.1 ± 2.22	48.9 ± 0.3

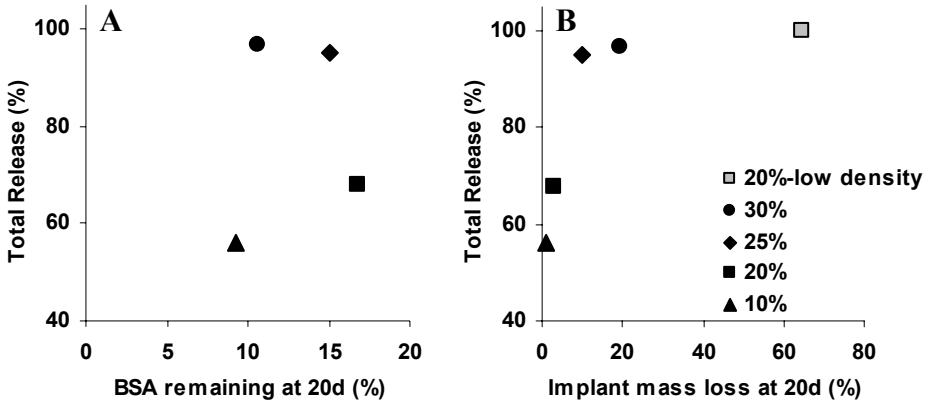

Figure 3.C.9 Total BSA release as a function of (a) the actual BSA content of the implants prior to the erosion and (b) the BSA-related implant mass-loss until day 20 as a measure of porosity (3 mm, 10 – 30% BSA loading, PBS pH 7.4); The low density implants (gray square) were prepared with ethanol-containing PLGA resulting in half the weight of the same sized implants

Besides an increase of the diffusional protein release (12, 30 and 40%), a decrease of the implant size from 10 over 7 to 3 mm resulted also in an increase (38, 45 and 55%) of the BSA fraction released during the polymer erosion phase (Table 3.C.1). The polymer erosion kinetic was not affected by the implant size as indicated by similar cumulative pH profiles (data no shown), which excluded its effect on the release completeness. A correlation between the diffusional and the erosional and hence the total BSA release, therefore, could be assumed. The rational behind it could potentially be either because of dependency of the PLGA-BSA interaction to the amount of protein present in the implant during polymer erosion or due to a decreased exposure of the protein to high oligomer concentrations in the implant governed by the porosity of the implants. A relation between the extent of the diffusional and the total release was previously seen for the release characteristics of implants with different BSA loadings (see chapter 3.B). Re-analysis of the release data of these implants revealed that there was no relation between the total release and the BSA amount present at the beginning of the erosion phase (Figure 3.C.9a), whereas a dependency of the release completeness on BSA-related implant mass-loss could be concluded (Figure 3.C.9b). The data suggested that in order to achieve complete protein release, PLGA implants should exhibit more than 10% porosity before the erosion phase starts.

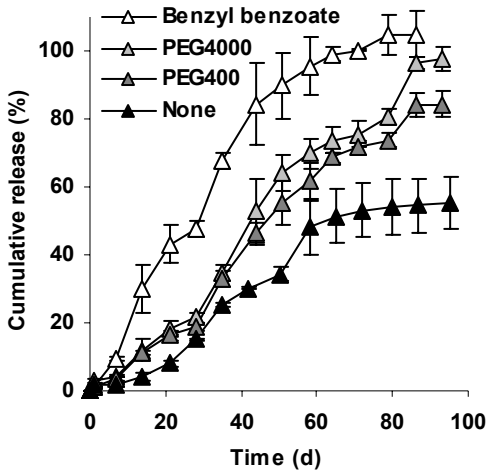

Figure 3.C.10 Effect of PLGA plasticizers (10% based on polymer) on the release of 10% BSA-loaded implants in PBS pH 7.4 (3 mm)

Pores in implants can be a product of the formulation process or be formed in situ during release. Addition of plasticizers could also increase the porosity and hence the total protein release through increasing the free-volume of the polymeric matrix. Accordingly, addition of the hydrophilic plasticizers PEG 400 and 4000 increased the release especially during the erosion phase (Figure 3.C.10). PEG can enhance drug release through its plasticization and pore-forming effects and at the same time suppress the diffusional release via increasing viscosity. In contrast to PEGs, hydrophobic benzyl benzoate increased BSA release during both diffusion- and erosion-controlled phases (Figure 3.C.10). However, both benzyl benzoate and PEG changed glass transition temperature of PLGA to a same extent (from ~ 47°C to 30°C) which suggested similar plasticization effect. Thus, the lower diffusional BSA release in presence of PEG was attributed to its ability to suppress the initial diffusion-controlled release of proteins (Bittner 1999; Ghalanbor et al. 2010). Accordingly, addition of benzyl benzoate resulted in complete BSA release as well as complete PLGA erosion through the increased porosity upon diffusional release in addition to the increased free-volume.

3.C.6 Conclusion

Formation of covalent adducts of BSA-PLGA was previously reported as a mechanism for incompleteness of the protein release (see chapter 3.B). This study proposed tools to increase BSA release completeness with special focus on PLGA degradation and erosion.

The presence of BSA reduced the PLGA degradation and erosion rate as well as the extent of erosion. On the other hand, the total releasable protein fraction was correlated with the extent of PLGA mass-loss.

Release study of implants prepared with pre-degraded PLGA suggested that induction phase was not responsible for release incompleteness. Thus, to achieve complete release, enhancement of protein release and out-flux of degradation products, through an increased porosity during erosion phase, was intended. The porosity could be a result of higher diffusional release e.g. by reduction of matrix size or increasing BSA loading above its percolation threshold. Moreover, the implants could be produced initially with lower density e.g. using ethanol-containing PLGA. Addition of pore-formers and/or plasticizers also increased both diffusional and erosional release. Consequently, no residual implant mass remained after complete release of BSA.

CHAPTER

4 Summary

Maintenance of protein stability during formulation processing, storage and release is one of the main challenges for the effective parenteral delivery of protein drugs. Parenteral delivery with biodegradable injectable delivery systems based on poly(lactide-co-glycolide) (PLGA) has been successfully applied for the controlled delivery of low-molecular-weight drugs and peptides. This was, however, less successful for protein drugs due to their higher structural and functional complexity. Since proteins stability is generally better in the solid-state, hot-melt extrusion (HME) as a solvent-free process was evaluated for processing of protein formulations. Yet, the high processing temperatures and pressures in HME can be potential stress factors for protein stability.

The purpose of this study was to assess the feasibility of hot-melt extrusion for preparing implants based on protein/PLGA formulations with special emphasis on protein stability, burst release and release completeness.

Model protein (lysozyme)-loaded PLGA implants were prepared with a screw extruder and a self-built syringe-die device as a rapid screening tool for HME formulation optimization. Lysozyme stability was determined using DSC, FTIR, HPLC and biological activity. Lysozyme was recovered from implants with full biological activity after HME. The effect of hydration upon contact with the release medium (as a potential protein instability factor) on the recovery was studied by extraction of lysozyme from the

implants after 1 day release. In spite of full recovery, functional stability was affected by the HME process conditions and its impact on the resulting matrix properties (e.g., porosity). Under optimized conditions, complete active recovery of the protein was obtained. The release from all implants reached the 100% value in 60–80 days with nearly complete enzymatic activity of the last fraction of released lysozyme. Pure PLGA implants with up to 20% lysozyme loading could be formulated without initial burst. To obtain optimized formulations with high drug loading and low burst release the simultaneous effect of lysozyme and PEG loadings on the initial release was investigated. This screening revealed that the effect of PEG is dependent not only on its size and concentration but also on the concentration of the protein. At drug loadings exceeding 20%, incorporation of PEG 400 reduced the initial burst. Accordingly, a complete lysozyme recovery in active form with a burst-free and complete release from PLGA implants prepared by hot-melt extrusion was obtained. This is in contrast to several reported microparticulate lysozyme-PLGA systems and suggests the great potential of hot-melt extrusion for the preparation of protein-PLGA implants.

The conformational stability during HME process was further examined with three less stable proteins; ovalbumin, cytochrome C and bovine serum albumin (BSA). DSC and FTIR revealed stability of the proteins and absence of non-covalent aggregation. This was especially interesting in the case of BSA which is a large and multi domain protein and highly prone to aggregation. MALDI-MS analysis revealed no detectable impurity formation after the process. BSA however, presented more challenges during release; high initial burst and incomplete release. The initial burst release was reduced by milling the protein prior to extrusion. Thus, PLGA implants with up to 25% BSA loading could be formulated without initial burst. The cumulative release was incomplete at 70% at loadings below the percolation threshold of the protein, while higher protein loadings increased the release to 97%. However, in all cases, an insoluble implant mass remained for over 6 months. Analysis of the residual matrices suggested a covalent linkage of BSA to PLGA oligomers via thioester bonds. Thus, covalent protein-PLGA adducts were responsible for the incomplete release from the delivery systems containing BSA. Increasing the porosity of the implants using ethanol-containing PLGA resulted in 100% release at 90 days with no insoluble implant mass remaining.

Nevertheless, the presence of PLGA in the insoluble implant mass was surprising because in the absence of proteins PLGA degraded completely into water-soluble oligomers within 50-60 days. BSA reduced the PLGA degradation and erosion rate and most importantly the extent of the erosion. The total releasable protein fraction was correlated with the extent of PLGA mass-loss. Release study of implants prepared with pre-degraded PLGA exhibited similar BSA release pattern and completeness. This suggested that the degradation phase was not responsible for release incompleteness. Thus, to achieve complete release, enhancement of out-flux of degradation products, through an increase in porosity during the erosion phase, was intended. The porosity could be a result of higher diffusional release e.g. by reduction of matrix size or increasing BSA loading above its percolation threshold. Addition of pore-formers and/or plasticizers also increased both diffusional and erosional release. Consequently, no residual implants remained after complete release of BSA. Accordingly, enhancement of out-flux of PLGA degradation products, once formed, was proposed to increase protein-release completeness.

In conclusion, hot-melt extrusion as a solvent-free process provided better stability of proteins and hence is a promising technique for processing of biodegradable implants for delivery of protein drugs.

CHAPTER

5 Zusammenfassung

Eine der größten Herausforderungen bei der Formulierung von Depotarzneiformen für Proteinwirkstoffe ist die Erhaltung der strukturellen und funktionellen Integrität der Wirkstoffe während der Herstellung, der Lagerung und der Freisetzung der Darreichungsform. Die Verabreichung von Arzneistoffen mit geringem Molekulargewicht und Peptiden mittels injizierbarer poly(glycolsäure-co-milchsäure)-basierter Depotformulierungen findet bereits breite Anwendung, welche den instabileren Proteinarzneistoffen bisher verwehrt blieb. Die ist unter anderem auf unvorteilhafte Einbettungsbedingungen zurückzuführen, welche die Proteinstruktur negativ beeinflussen können. Proteine weisen im festen Zustand eine generell höhere Stabilität gegenüber denaturierenden Einflüssen auf als in Lösung. Die Schmelzextrusion erlaubt die lösemittelfreie Einbettung von Proteinpartikeln in bioabbaubare Polymermatrices. Die dabei potenziell auftretenden hohen Drücke und Prozesstemperaturen sind allerdings mögliche Stressfaktoren für Proteine

Mit dieser Arbeit sollte die Anwendbarkeit der Schmelzextrusion für die Herstellung von proteinbeladenen Implantaten auf Basis des bioabbaubaren Trägermaterials PLGA ermittelt werden. Schwerpunkte der Arbeit lagen dabei auf der Aufrechterhaltung der Proteinstabilität während der Herstellung, dem Erreichen einer vollständigen Proteinfreisetzung und dem Vermeiden einer hohen initialen Freisetzung.

PLGA-Implantate mit Lysozym als Modellwirkstoff wurden mit einem 2-Schneckenextruder hergestellt. Ein selbstgebautes Instrument zur Extrusion kleiner Chargengrößen wurde zum Screening für HME Formulierungen verwendet. Die Lysozym-Stabiltät wurde mit DSC, FTIR, HPLC und über die biologische Aktivität ermittelt. Nach der Schmelzextrusion wurde Lysozym mit voller Aktivität wiedergewonnen. Um den Effekt der initialen Hydratation durch das Freisetzungsmedium auf die Proteinstabilität zu untersuchen, wurde Lysozym nach einem Tag Inkubation aus den Implantaten extrahiert. Trotzdem das Lysozym komplett wiedergewonnen werden konnte, wurde die Aktivität des Enzyms durch den HME-Prozess beziehungsweise dessen Einfluss auf die resultierende Matrixporosität verringert. Unter optimierten Bedingungen konnte der gesamte Proteinwirkstoff mit voller Aktivität aus den Implantaten wiedergewonnen werden. Die Freisetzung war aus allen Implantaten nach 60-100 Tagen vollständig wobei nahezu 100%ige enzymatische Aktivität nachgewiesen werden konnte. Reine Protein/PLGA Implantate konnten mit bis zu 20% Lysozym beladen werden ohne dabei eine hohe initiale Freisetzung (Burst) zu verursachen. Um Formulierungen mit hoher Arzneistoffbeladung und geringem Burst zu erhalten wurde der simultane Einfluss von PEG- und Lysozymbeladung der Implantate auf die initiale Freisetzung untersucht. Dieses Screening zeigte einen Effekt des PEG auf die Freisetzung der sowohl auf dessen eigener Konzentration, der Molekülgröße und auch auf der Konzentration des Proteins beruhte. Bei mehr als 20% Arzneistoffbeladung reduzierte PEG 400 den initialen Burst. Dadurch konnte eine Freisetzung von aktivem Lysozym ohne eine hohe initiale Freisetzung erreicht werden. Dies steht im Gegensatz zu vielen in der Literatur beschriebenen bioabbaubaren Mikropartikelsystemen. Die Schmelzextrusion erschien demnach zur Herstellung von Protein-PLGA-Implantaten geeignet.

Es wurde weiterhin getestet, ob drei Proteine mit geringerer Stabilität während des HME-Prozesses intakt bleiben würden; Ovalbumin, Cytochrom C und Bovines Serumalbumin (BSA). DSC und FT-IR zeigten keine Zeichen für Denaturierungen oder die Präsenz von nicht-kovalenten Aggregaten, was auf die Erhaltung der nativen Konformation hindeutete. Besonders interessant war dies in Bezug auf BSA, da dies ein sehr großes, und viele Domänen beinhaltendes Protein ist, das besonders leicht aggregiert. Eine MALDI-MS Analyse zeigte keine nachweisbaren Verunreinigungen nach dem Prozess. Allerdings führte die Einbettung von BSA zu Schwierigkeiten bei der Freisetzung, welche

durch einen hohen initialen Burst und eine unvollständige Freisetzung gekennzeichnet war. Der initiale Burst konnte vermindert werden indem die Partikelgröße des Proteins vor der Extrusion verringert wurde. Danach konnten PLGA-Implantate mit bis zu 25% BSA Beladung hergestellt werden ohne einen Burst zu zeigen. Die Freisetzung war mit maximal 70% unvollständig, wenn BSA die BSA-Beladung unter der Perkolationsgrenze gehalten wurde. Wurde allerdings eine Beladung oberhalb der Perkolationsgrenze eingesetzt, stieg die kumulative Freitsetzung auf bis zu 97%. In allen Fällen blieb unlösliche Implantatmasse über einen Zeitraum von 6 Monaten bestehen. Die Analyse des Rückstandes lies auf eine kovalente Bindung des BSA mit PLGA-Oligomeren durch Thioesterbindungen schließen. Die Bildung von kovalenten Bindungen während der Erosionsphase war daher für die unvollständige Freisetzung von BSA aus den PLGA-Implantaten verantwortlich. Eine Erhöhung der Porosität der Implantate durch Ethanolbeimengung zu der zu extrudierenden Protein-/Polymermasse, führte zu einer vollständigen BSA Freisetzung innerhalb von 90 Tagen, ohne dass sich ein unlöslicher Rückstand bildete.

Überraschend war, dass PLGA in dem unlöslichen Rückstand nachgewiesen werden konnte, da PLGA in Abwesenheit von Proteinen innerhalb von 50-60 Tagen in wasserlösliche Oligomere abgebaut wird. BSA verlangsamte den PLGA-Abbau und die Erosionsrate leicht und verringerte insbesondere das Ausmaß der Erosion. Freisetzungsversuche mit Implantaten aus pre-degradiertem PLGA zeigten ähnliche Proteinfreisetzungsprofile und Ausmaße der Freisetzung im Vergleich zu unverändertem Polymer. Daraus konnte geschlossen werden, dass die Phase des rein chemischen Abbaus des PLGA nicht verantwortlich für die unvollständige Freisetzung des Proteins war. Als Konsequenz wurde eine Erhöhung des out-flux der Abbauprodukte angestrebt, um eine komplette Freisetzung zu erreichen. Dies sollte durch Erhöhung der Porosität des Implantats in der Erosionsphase erreicht werden. Um die Porosität zu erhöhen, gab es mehrere Möglichkeiten: erleichterte diffusionskontrollierte Freisetzung durch eine Verkleinerung der Matrix oder eine Erhöhung der BSA-Beladung über die Perkolationsgrenze hinaus. Auch ein Zusatz von porenformenden Stoffen und/ oder Weichmachern konnte die diffusions- sowie die erosionsbedingte Freisetzung erhöhen. Infolgedessen blieb nach vollständiger BSA-Freisetzung kein unlöslicher Rückstand

zurück. Man kann daraus schließen, dass die Beschleunigung des Out-flux des löslichen PLGAs, die vollständigere Freisetzung von inkorporierten Proteinen zur Folge hat.

Die Schmelzextrusion bietet folglich die Möglichkeit, Proteine ohne den Einsatz von Lösemitteln in bioabbaubare Polymermatrices einzubetten ohne die Stabilität negativ zu beeinflussen. Es ist deshalb eine sehr vielversprechende Technik um bioabbaubare Implantate für Proteinwirkstoffe herzustellen.

CHAPTER

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CHAPTER

7 Publications and Presentations Resulting from This Work

Journal Publications

Z. Ghalanbor, M. Körber, R. Bodmeier, *Improved Lysozyme Stability and Release Properties of Poly(lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion*, Pharmaceutical Research, 2010: 27(2), 371-379.

Z. Ghalanbor, M. Körber, R. Bodmeier, *Protein Release from Poly(lactide-co-glycolide) Implants Prepared by Hot Melt Extrusion: Thioester Formation as Reason for Incomplete Release*, (Submitted manuscript).

Z. Ghalanbor, M. Körber, R. Bodmeier, *Interdependency of Protein-Release Completeness and Polymer Degradation in PLGA-Based Implants*, (Submitted manuscript).

Poster Presentation

Z. Ghalanbor, M. Körber, and R. Bodmeier, *BSA Stability and Release from Hot Melt Extruded Poly(lactide-co-glycolide) Implants*. "The 37th Annual Meeting and Exposition of The Controlled Release Society", 2010, Portland, Oregon, USA.

CHAPTER 8

Curriculum Vitae

For reasons of data protection, the Curriculum Vitae is not included in the online version

Publications

2010 F. Schmidt, Z. Ghalanbor, F. Thormann, M. Kühbacher, R. Bodmeier, U. Gross, H. Schubert, R. Zehbe, *Drug Loaded, Biodegradable Nerve Conduits for the Simultaneous Chemical and Electrical Stimulation of Neural Cells as a Therapeutic Approach for Peripheral Nerve Regeneration*, Advanced Materials Research, 89-91: 497-502.

2008 Z. Ghalanbor, N. Ghaemi, S.-A. Marashi, M. Amanlou, M. Habibi-Rezaei, K. Khajeh, B. Ranjbar, *Binding of Tris to Bacillus Licheniformis α-Amylase Can Affect its Starch Hydrolysis Activity*, Protein and Peptide Letters, 15: 212-214.

2006 M. Rezaei-Tavirani, S.-A. Marashi, Z. Ghalanbor, M. Mostafavi, *Proteomics*, (ISBN 964-96805-0-0).

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2004 S.-A. Marashi, Z. Ghalanbor, *Correlations Between Genomic GC Levels and Optimal Growth Temperatures Are not 'Robust'*, Biochemical and Biophysical Research Communications, 325: 381-383.